Tampere University

Iida-Elisa Launonen

IN VITRO HEPATOCYTE CO-CULTURE METHODS

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TIIVISTELMÄ

Iida-Elisa Launonen: *In vitro* -hepatosyyttien yhteisviljelymenetelmät
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Tämän kirjallisuuskatsauksen tarkoituksena oli selvittää, voivatko 3D-soluviljely ja yhteisviljely muiden solutyyppien kanssa parantaa ihmisen pluripotenteista eli erittäin monikykyisistä kantasoluista erilaistettujen hepatosyyttien kypsyysastetta. Kyseiset kantasolut ovat saaneet viimeisen vuosikymmenen aikana paljon huomiota, sillä niistä on opittu tuottamaan helposti ja nopeasti lähestulkoon mitä tahansa ihmisen soluja tutkimuksen käyttöön. Näiden erilaistettujen solujen ongelmaksi on kuitenkin muodostunut *in vivo* -ihmiskudoksen soluja huonompi toiminnallisuus. Tätä ongelmaa on lähdetty ratkomaan mm. viljelyolosuhteita optimoimalla.

Tutkimusaineistona toimivat artikkelit kerättiin PubMed-tietokannasta kesällä 2020 – syksyllä 2021. Aineistoksi hyväksyttiin artikkelit, jotka käsittelivät joko uudelleenohjelmoiduista kantasoluista, tai normaaleista ihmisestä eristetyistä kantasoluista erilaistettujen hepatosyyttien edellä mainittujen viljelymenetelmien tuloksia. Valitut artikkelit jaettiin kolmeen ryhmään viljelymenetelmän mukaan: 3D-soluviljellyt, yhteisviljellyt ja 3D-yhteisviljellyt eli organoidit. Artikkelien raportoimat löydökset kypsyysasteesta ryhmiteltiin menetelmien mukaan eritellen havainnot morfologian, albumiinin tuoton, urean erityksen, triglyseridien ja lipoproteiinien, lääkeaineita hajottavien entsyymien ja muiden geneettisten tekijöiden mukaan.

Tutkimuksen tuloksista havaitaan, että 3D-yhteisviljely paransi erilaistettujen hepatosyyttien kypsyysastetta parhaiten: ainoastaan näissä viljelmissä esiintyi hiussuonia muistuttavia rakenteita, albumiinia tuotettiin vähintäänkin verrattavissa olevat määrät *in vivo* -hepatosyytteihin nähden ja solujen geeniekspressio vastasi parhaiten tavoiteltua *in vivo* -hepatosyyttien geeniekspressiota. Vertailtaessa pelkästään 3D-viljelyä ja yhteisviljelyä toisiinsa, tuloksista voidaan päätellä 3D-viljelyn parantavan erilaistettujen hepatosyyttien kypsyysastetta suhteessa enemmän. Tuloksia tarkastellessa on kuitenkin huomioitava artikkeleiden kontrolloimattomat tutkimusasetelmat ja vähäiseksi jäänyt määrä, mitkä lisäävät satunnaisvirheen mahdollisuutta tulkintoihin.

Avainsanat: iPS-solut, organoidit, hepatosyytit, kypsyysaste, 3D-viljely, yhteisviljely

ABSTRACT

There's an incremental demand for functional hepatocytes *in vitro* to be used for a variety of medical applications. While differentiated adult cells such as human hepatocytes quickly lose their mature phenotype and functionality *in vitro*, human pluripotent stem cells (hPSCs) have yielded more promising results. However, the state of maturity of these hPSC-derived hepatocytes still tends to be slightly impaired when compared to *in vivo* hepatocytes highlighting the need for better culturing techniques.

This paper reviews firstly the morphological, structural, and physiological differences between immature and mature hepatocytes, and secondly two current strategies, namely co-culturing and 3D setting, to improve the maturation state of human pluripotent stem cell-derived hepatocytes. As a major finding, combining these techniques as 3D co-culturing and producing organoids yields the best results in terms of the maturity measures used in this review. 3D co-culture was the only culturing method to provide capillary-like structures, albumin production even higher than in primary human hepatocytes (PHHs) *in vivo*, as well as highly comparable gene signatures for triglyceride & lipoproprotein metabolism, HNF4α transcript and protein levels all in the range of PHHs.

While organoids were in fact superior in generating the most mature hPSC-derived hepatocytes in this review, comparing the results of different study settings is difficult and questionable to say the least. This text highlights the need for further research, as there are multiple other factors affecting the maturity of cultured hepatocytes apart from the culturing method.

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1 A BRIEF INTRODUCTION

Cell-based therapies for liver diseases, liver drug testing and
liver disease modelling are just a few examples of medical applications requiring continually growing amounts of functional hepatocytes. Human pluripotent stem cells have received attention as an encouraging source to meet the demand for these hepatic cells. However, the problem lies in the immature state of these human pluripotent stem cell-derived hepatocytes.

This paper reviews firstly the morphological, structural, and physiological differences between immature and mature hepatocytes, and secondly two current strategies, namely co-culturing and 3D setting, to improve the maturation state of human pluripotent stem cell-derived hepatocytes. The text highlights the need for further research, as the more we find usable hepatic differentiation methods, the more questions rise on their underlying mechanisms.

2 HUMAN PLURIPOTENT STEM CELLS

Human pluripotent stem cells (hPSCs) are cells with the ability to indefinitely self-renew and to differentiate into derivatives of all three germ layers, namely endoderm, mesoderm and ectoderm. hPSCs include human embryonic stem cells (hESCs) and more recently developed human induced pluripotent stem cells (hiPSCs) (Thomson, 1998; Takahashi et al., 2007). Both cell types provide an unlimited cell source for regenerative medicine applications and may be utilized in studying genetic diseases and developmental processes. Until their discovery, different diseases were mostly modelled using animals and engineered cell lines, often with poorly correlating results to human studies. In addition, obtaining functional human tissue from patients is difficult and differentiated adult cells such as primary human hepatocytes (PHHs) quickly lose their mature phenotype *in vitro* (Nguyen et al., 2016). Therefore, there are high hopes placed on the future for hPSCs, particularly for hiPSCs, as they contain patient's genetic mutations and thus can be utilized in the research on genetic diseases.

hESCs were first derived in 1998 by Thomson and co-workers (Thomson, 1998). Often, the inner cell mass of a blastocyst serves as a source for hESCs but morula staged embryos may also be utilized (Damdimopoulou et al., 2016). Induced pluripotent stem cells, in turn, were reprogrammed and reported for the first time in 2006 by Yamanaka and co-workers (Takahashi & Yamanaka, 2006). They reported a way to reprogram fully differentiated mouse cells back to the pluripotent stage using retroviral induction with specific pluripotency factors *OCT3/4*, *SOX2*, *KLF4* and *C-MYC.* These transcription factors enabled the pluripotency genes found in the differentiated cells to be turned back on and thus induced the pluripotent state. The next year, the team repeated the

reprogramming process using human fibroblasts, consequently giving rise to hiPSCs (Takahashi et al., 2007). As this method involves risks of tumorigenicity, uncontrolled gene expression and potential reactivation of the virus, a variety of new methods using non-viral and/or non-integrative methods have been reported, e.g. episomal plasmids and nucleofections (Okita et al., 2011; Paulitschek et al., 2017). Fibroblasts have remained as a popular donor cell type, and along them blood cells are also an easy choice due to their availability.

The understanding on hPSCs and their differentiation into different cell types has progressed to the point where we are able to produce most of the human cell types in the laboratory. Currently popular hepatic differentiation methods from hPSCs include the combinational transduction of *FOXA2* and *HNF1α*, or *SOX17*, *HEX* and *HNF1α*, in addition to a treatment with appropriate growth factors and cytokines (Si-Tayeb et al., 2010; Song et al., 2009). In general, most differentiation protocols proceed from stem cell phase to hepatoblast stage to hepatocyte-like cells (HLCs). hPSC-derived hepatocytes are generated for disease research and drug development, and they're beginning to enter the clinical research phase for the testing of liver regeneration therapies (Corbett & Duncan, 2019). However, a major problem regarding their use is the immature state of these type of hepatocytes. Most protocols used to differentiate different type of cells from hPSCs generate cells at embryonic or early fetal stages, and thus the generated cells lack many qualities of adult cells. Some of these qualities are fundamental in regard of the use of hPSC-derived cells in modelling of adult-onset diseases, drug screening and replacing cells lost to disease. As many liver, and other, diseases have middle-age onset, these limitations are relevant for research.

3 LIVER AND HEPATOCYTES

As one of the largest organs in the body, liver performs multiple tasks including some crucial metabolic, synthetic, immunologic and detoxification processes. These include but are not limited to carbohydrate & lipid metabolism, bile acid & urea synthesis, and drug & waste product detoxification. (Trefts et al., 2017). The main parenchymal cells of the liver, also known as hepatocytes, make up approximately 80% of the liver with the remaining 20% constituting of different non-parenchymal cells: biliary epithelial cells, sinusoidal endothelial cells, hepatic stellate cells, Kupffer cells and pit cells (Kmiec, 2001). In accordance with the functions of the liver, hepatocytes are responsible for the production of albumin, urea, triglycerides, lipoproteins and enzymes for drug metabolism. Biliary epithelial cells, or cholangiocytes, line the biliary tract within the liver and release bicarbonate and water to the bile. Layers of sinusoidal endothelial cells join to form walls of capillaries. They control the fat uptake of the liver and participate in the functions of the reticuloendothelial system. Hepatic stellate cells, also known as lto cells or fat-storing cells, are

thought to be the main producers of extracellular matrix (ECM) components. Kupffer cells, in turn, are liver tissue macrophages with the ability to mediate the inflammatory response and take part in the immune defense. Finally, pit cells are natural killer cells specific to the liver. (Kmiec, 2001). Figure 1 depicts these cell types as a part of a sinusoid.

The microstructure of the liver is complex but highly organized. Hepatocytes are separated from sinusoids via spaces of disse and fenestrated endothelial cells. Stellate cells in turn situate between the endothelial cells and hepatocytes. Kuppfer and pit cells are often found in the sinusoids, although they are free to roam around the tissues. (Kmiec, 2001; Nakatani et al., 2004). These non-parenchymal cells participate in producing various types of environmental signals, such as soluble factors, ECM components and cell-cell interactions, which all seem to be crucial in terms of hepatocytes functioning properly. As the microenvironment changes when i.e., transferring the parenchymal cells to *in vitro* setting, the cells dedifferentiate rapidly (Kiamehr et al., 2019). Consequently, there has been a growing interest in performing the hepatocyte differentiation and maturation process *in vitro*, and for that purpose it is crucial to know some major hepatocyte maturation markers. Discussed in the next section, these maturation markers may be utilized in deducing the maturation state of the hepatocytes.

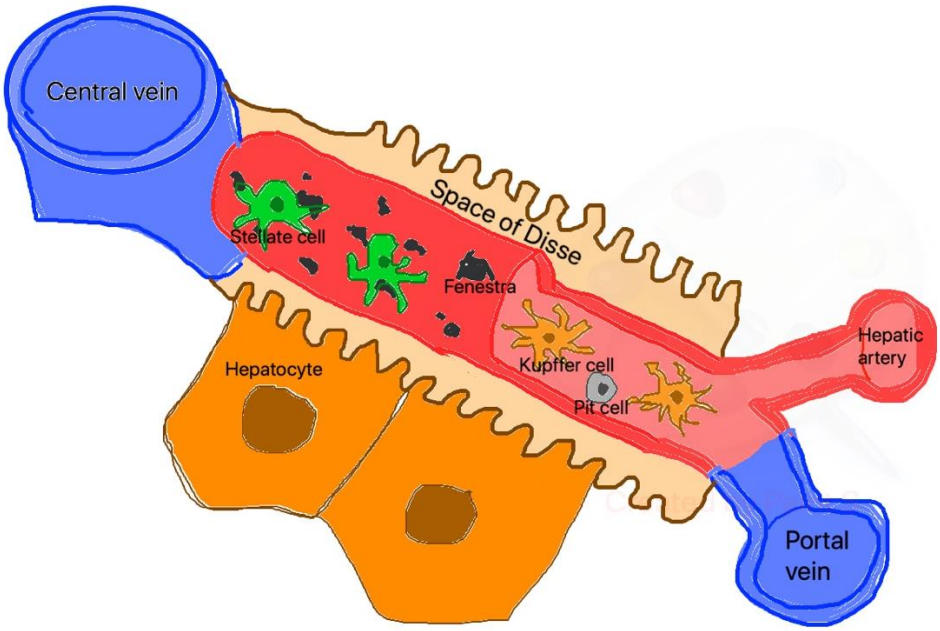

Figure 1. The microstructure of a sinusoid excluding bile ducts and canaliculi. An adapted and modified version (Nahmias, n.d.).

4 ESTIMATING HEPATOCYTES MATURITY STATE

As stated, traditionally cultured hPSC-derived hepatocytes tend to reach the maturation state of fetal tissue instead of the desired, fully matured freshly isolated primary human hepatocytes (PHHs). Hence it is time worthy to consider some main differences between these two stages and what kind of structural and functional variance they may bring about. Since the hepatic differentiation protocols, evaluation techniques and functional outcomes vary between different laboratories, the comparison is at times rather challenging.

While there is a lot of data on the expression levels of specific encoding genes, it is crucial to bear in mind that they don't always correlate with the actual activity levels of the produced proteins. However, often they do point in the right direction. When comparing the expression levels of enzymes in hepatocytes during the fetal and mature states, most enzymes may be roughly divided into three groups. The first group contains enzymes, e.g. CYP3A7, with expression levels decreasing from fetal to postnatal period. Enzymes in the second group, such as UGT1A1, present low expression levels during the fetal state but show a blow-up at birth and then gradually increase with time to adult levels. Finally, the third group enzymes, e.g. CYP1A2 and CYP2B6, have low expression levels until the neonatal period and progress to express mature levels years after birth. (Zabulica et al., 2019) The goal is to be able to produce hPSC-derived hepatocytes with similar enzymatic activities to fully matured *in vivo* ones, and one way of roughly monitoring this is comparing the expression levels of the corresponding encoding genes.

4.1 Morphology

With current 2D monoculture differentiation strategies, it has been possible to generate hPSC-derived hepatocytes which resemble the morphology of mature hepatocytes rather well. The differentiated cells have a polygonal shape, present dense and up to 16% binucleated nuclei and sufficiently high cytoplasm to nuclei ratio (Kiamehr, 2019). PHHs have microvilli on cell surface which conventionally differentiated hPSC-derived hepatocytes present, too. However, there is room for improvement with the distribution of the proteins. In other words, the degree of polarization in hPSC-hepatocytes is significantly smaller than in PHHs. Furthermore, endoplasmic reticulum were also found to be poorly organized in traditional cultures of hPSC-derived hepatocytes. (Thi et al., 2020).

4.2 Albumin secretion

The production of albumin is one of the most important functions of hepatocytes. Holmgren and colleagues found albumin to be expressed as mRNA and secreted as a protein in hPSC-derived hepatocytes at similar levels to PHHs (Holmgren et al., 2020). In addition, according to a study conducted by Zabulica and colleagues, there is virtually no difference in *ALB* (albumin encoding gene) expression levels between fetal and mature livers (Zabulica et al., 2019). However, as reported by a study by Baxter et al., the hPSC-derived hepatocytes secreted albumin at levels which were at least comparable to those from fetal hepatocytes but 8-fold lower than those from PHHs (Baxter et al., 2015). Furthermore, as is stated later in this literature review, some studies have showed increased levels of albumin expression and secretion with improved cultivation methods (co-culture and 3D environment) when compared to controls. Thus, one can deduce that merely differentiating hPSCs to hepatocytes won't result in optimal albumin secretion.

4.3 Urea production

While urea synthesis is a hepatocyte-specific function, urea secretion alone does not prove the activity of the entire urea cycle. Holmgren and colleagues estimated the functionality of the hPSC-derived hepatocytes' urea cycle by comparing the mRNA levels of several different cycle-related genes and secreted urea levels after ammonium challenge to the values obtained by freshly isolated PHHs. While some genes were expressed at comparable levels between these two cell types, genes such as *ARG1*, *ASL*, *ASS1*, *CPS1*, *OTC* and *SLC25A2* showed significantly lower expression levels in the hPSC-derived hepatocytes. Furthermore, possibly due to the lower expression of *ARG1*, also urea secretion was lower in the hPSC-derived hepatocytes. (Holmgren et al., 2020). More specifically, Baxter and colleagues reported on urea secretion by hPSC-derived hepatocytes to be comparable to fetal hepatocytes but 18-fold lower than PHHs (Baxter et al., 2015).

4.4 Triglycerides and lipoproteins

Regulating cholesterol levels in the body is another important function of hepatocytes. This is done by synthesizing and secreting lipoproteins into the blood and removing excess by converting it to bile acids. Holmgren and colleagues assessed the mRNA expression of 76 genes related in cholesterol and fatty acid metabolism. According to Holmgren's group and a few other studies, the majority of these genes were expressed at a mature, or PHH level, including LDL receptor (*LDLR*) and apolipoprotein B (*APOB*) (Holmgren et al., 2020; Schöbel et al., 2018; Takacs et al., 2017). Four of the cholesterol related genes assessed by Holmgren et al. were, however, found to differ

significantly from mature hepatocytes: ATP citrate lyase (*ACLY*), apolipoprotein A1 (*APOA1*), apolipoprotein A4 (*APOA4*) and apolipoprotein A5 (*APOA5*). The first three were reported to be expressed at higher levels in hPSC-derived hepatocytes than in PHHs, while *APOA5* was expressed at lower levels. With fatty acid metabolism genes, three were expressed at differing levels in hPSC-derived hepatocytes when compared to mature ones: acetyl-CoA carboxylase beta (*ACACB*), bile acyl-CoA synthetase (*SLC27A5*) and protein kinase AMP-activated catalytic subunit alpha 2 (*PRKAA2*). (Holmgren et al., 2020).

4.5 Drug metabolizing enzymes

Human cytochrome P450 genes, also known as CYP genes, are crucial regarding the metabolism of both endogenous and exogenous components. There has been a lot of research on their role in drug metabolism, and the coherent results indicate that the proper functioning of CYPs is crucial for hepatocytes. Except for CYP1A1, Zabulica et al. reported that hPSC-derived hepatocytes tend to express CYP genes comparable to the lower levels of fetal tissue in contrast to mature hepatocytes. In detail, the expression levels of *CYP1A2*, *CYP2C8* and *CYP2D6* were found to be in the range of fetal tissue, while *CYP2C9*, *CYP2C19* and *CYP2B6* were expressed at levels between the ones observed in fetal and mature hepatocytes. (Zabulica et al., 2019) Holmgren and colleagues got roughly similar results with *CYP1A2*, *CYP2B6* and *CYP2D6* found to be expressed at more immature levels, while *CYP2C9* and *CYP2C19* showed comparable expression levels to PHHs (thought as mature tissue) (Holmgren et al., 2020). They also assessed the enzyme activity, describing mature CYP1A, CYP3A and CYP2C9 and immature CYP2B6 and CYP2D6 activities (Holmgren et al., 2020).

Perhaps the best known CYPs in terms of maturity, *CYP3A4* and *CYP3A7*, have often been referred to as the adult and fetal forms of *CYP3A*, respectively (Betts et al., 2015). Zabulica and colleagues reported that hPSC-derived hepatocytes were able to acquire the low levels of *CYP3A4* similar to mature hepatocytes though they still express *CYP3A7* at levels comparable to fetal hepatocytes (Zabulica et al., 2019). Coherently, Baxter and colleagues reported on the protein levels for CYP3A to be 10 % of the levels presented by PHHs (Baxter et al., 2015). Holmgren's group, however, found both *CYP3A4* and *CYP3A7* to be expressed comparably to PHHs (Holmgren et al., 2020).

4.6 Other genetic components

Alpha-fetoprotein (AFP) is often used as one specific indicator of the maturation state of hepatic cells. The lower its amount, the more mature the cell is thought to be, i.e. the expression levels of AFP are higher in fetal compared to mature liver hepatic tissues. (Zabulica et al., 2019). While fetal and mature tissues seem to express comparably similar levels of genes encoding alpha-1-antitrypsin (*A1AT*), glutamate-ammonia ligase (*GLUL*), carbamoyl-phosphate synthase 1 (*CPS1*), phenylalaninen hydroxylase (*PAH*) and fumarylacetoacetate hydrolase (*FAH*), hPSC-derived hepatocytes have not reached these levels. In addition, ornithine carbamoyltransferase (*OTC*) expression levels were found to be lower in both fetal and hPSC-derived hepatic tissues when compared to mature tissue. (Zabulica et al., 2019). However, Holmgren and colleagues investigated the expression of some other hepatocyte-markers, namely asialoglucoprotein receptor 1 (*ASGPR1*), connexin 32 (*GJB1*) and α1-antitrypsin (*AAT*), and they were found to be expressed at similar mRNA levels when compared to PHHs (Holmgren et al., 2020).

A group of nuclear hormone receptors referred to as hepatocytes nuclear factors (HNFs) have also expressed lower fetal levels in hPSC-derived hepatocytes when compared to the levels expressed by mature hepatocytes (Zabulica et al., 2019). However, according to another study, the gene $HNF4\alpha$ was found to be expressed at similar mRNA levels as in PHHs. The same study also showed almost all hPSC-derived hepatocytes to be immune-positive for HNF4α. (Holmgren et al., 2020).

5 IMPROVING THE MATURITY OF IPSC-DERIVED HEPATOCYTES

Research has found multiple different components which promote hepatocyte differentiation and maturation. Growth factors, transcription factors, microRNAs, small molecules and finally the microenvironment are all examples of these differentiation boosting components (Xie et al., 2021). This literature review focuses on the lastly mentioned, namely microenvironment, and more specifically on exposing hPSC-derived hepatocytes to co-cultures and three-dimensional cultures. These methods as well as their maturational effects are reviewed and compared, still bearing in mind that different studies have been performed with different regimes, standards, and equipment.

5.1 2D co-cultures of hPSC-hepatocytes with non-parenchymal cells

Co-culturing of human hepatocytes with other cell types has been done for decades. In fact, in 1984 a study was published on the influence of rat epithelial cells being cultured with hepatocytes (Clement et al., 1984). Since its success in slowing down the dedifferentiation of the freshly isolated

hepatocytes, countless other study groups have shown the advantages of co-culturing with not only non-parenchymal cell types (epithelial of liver (Mertens et al., 1993), stellate (Abu-Absi et al., 2004), sinusoidal endothelial (Bale et al., 2015) and Kuppfer cells (Zinchenko et al., 2006b)) but also to some extent with non-liver derived cell types (epithelial of lungs and kidneys (Donato et al., 1991)). Furthermore, these cells don't necessarily need to be from human (Bhatia et al., 1999). The positive effects found were reported to stem from the growth, migration, and differentiation of cells, which in turn were provoked by the heterotypic cell-cell interactions. In fact, these interactions have been found to be highly significant already in during the fetal period, especially with foregut endodermal cells and endothelial cells (Houssaint, 1980; Matsumoto et al., 2001).

Naturally, hopes are high for co-culturing to also improve the maturity of hPSC-derived hepatocytes. To test this hypothesis, multiple different co-culturing techniques with well-regulated heterotypic cell-cell and cell-ECM interactions have been introduced. New methods are of course developed continuously. Before going through what kind of maturational changes in hPSC-derived hepatocytes have been established by their use, first the co-culturing methods themselves will be reviewed.

5.1.1 Methods of 2D co-culturing

Micropatterning is thought to be one of the most traditional way to examine the effect of co-culturing on hPSC-derived hepatocytes (Zinchenko et al., 2006a). Other options include using micro-bioreactors and microfluidic biochips for a dynamic culture and thus improved phenotype and stacking of cell sheets to also bring 3D culturing to question (Kehtari et al., 2018). As the combination of co- and 3D-culturing will be discussed later in this review, these type of research articles have been left out from this section.

Co-culturing as such has many different aspects to consider. Some research groups have decided to investigate paracrine signaling between the different cell types and so have cultivated hPSC-derived hepatocytes in the medium of the other cell type (Takagi et al., 2017). On the other hand, other investigations have had even tenfold amount of co-culturing cells per one hPSC-derived hepatocyte (Berger et al., 2015). Reportedly there have also been efforts to mimic the situation *in vivo* by having multiple different cell types co-cultured with the hPSC-derived hepatocytes (G. Wang et al., 2018).

5.1.2 Maturational effects of 2D co-culturing

For the sake of clarity, the maturational advantages caused by co-culturing will be discussed in the same subsections which were introduced earlier on in this literature review. There were only few articles on mere 2D co-culturing of hPSC-derived hepatocytes with another human cell type and thus this section is quite brief.

Morphology

As expected, co-cultured hPSC-derived hepatocytes consistently presented a more mature morphology than their monocultured counterparts. Danoy and colleagues reported their co-cultures with hPSC-derived liver sinusoidal endothelial cells exhibiting *in vivo* type of networking and tubular-like structures unlike monocultures (Danoy et al., 2021). Another report on co-cultures with immortalized hepatic stellate cells stated, that hPSC-derived hepatocytes showed the morphology comparable to *in vivo* human hepatocytes. More specifically, this included polygonal shape, prominent nuclei, scant cytoplasm and enriched cytoplasmic granules. (Javed et al., 2014).

Albumin secretion

The secretion of albumin was the most frequently measured quantity in the studies. Albumin expression and secretion were found to be statistically significantly increased in co-cultures compared with monocultures with no exception. These co-cultures consisted of hPSC-derived hepatocytes with one of the following: hPSC-derived hepatic stellate cells, umblical vein endothelial cells, mesenchymal stem cell medium (no actual cells), hPSC-derived liver sinusoidal endothelial cells or hepatic stellate cells. (Danoy et al., 2021; Javed et al., 2014; Kehtari et al., 2018; Miyoshi et al., 2019; Takagi et al., 2017) The albumin production in co-cultures with hPSC-derived liver sinusoidal endothelial cells was found to even reach the levels of PHHs *in vitro*. However, albumin production remained at least 3-fold lower than the *in vivo* estimates. (Danoy et al., 2021).

Urea secretion

The awaited increase in urea secretion in co-cultures was not as clear as with albumin. For example Kehtari et al. stated that the addition of umbilical vein endothelial cells to hPSC-derived hepatocytes in micro-bioreactor led to increased urea secretion, even though the difference was not significant (Kehtari et al., 2018). In a similar manner, Takagi's and Javed's research groups found the urea secretion in their co-cultures to be enhanced with uncertain significance (Javed et al., 2014; Takagi et al., 2017).

Triglycerides and lipoproteins

Unfortunately, none of the found articles had reported on the effect that co-culturing has on the triglyceride and lipoprotein levels. However, related to this field, Danoy et al. stated that the concentration of lipids was found to be higher in the co-culture with hPSC-derived liver sinusoidal endothelial cells compared to monoculture. (Danoy et al., 2021).

Drug metabolizing enzymes

Reports on the effect of co-culture on CYPs were a little incoherent. Takagi and colleagues stated that CYP3A4 activity was enhanced in hPSC-derived hepatocytes which had been conditioned in mesenchymal stem cell medium (Takagi et al., 2017). Another study found the expression of CYP7A1 to be more prominent in co-culture with umblical vein endothelial cells (Kehtari et al., 2018). However, Danoy et al. reported that no significant differences were found between the activities of CYP1A2, CYP2A6, CYP2C9 and CYP3A4 in co- vs monocultures, where co-cultures included liver sinusoidal endothelial cells (Danoy et al., 2021).

Other genetic components

The relative levels of AFP and HNFα expression in co-cultures were found to vary by study. Takagi and colleagues found *AFP* expression to be enhanced in mesenchymal stem cell medium, while Danoy et al. reported on similar amounts of expressed *AFP* and *HNF*α when compared between mono- and co-culture with liver sinusoidal endothelial cells (Danoy et al., 2021; Takagi et al., 2017). However, indicating an immature state, Javed and colleagues reported on *AFP* expression to be still detectable after 16 days in co-culture with immortalized hepatic stellate cells (Javed et al., 2014).

5.2 3D monocultures of hPSC-hepatocytes

While 2D culture for hPSC-derived hepatocytes has its own benefits in terms of ease of use, on the downside it seems to prevent the cultivated cells from organizing naturally in *in vivo* resembling 3D formation – and thus has a negative effect on the differentiation process and cell function (Schyschka et al., 2013). In addition, there is evidence for the culturing of once isolated PHHs in a 3D format causing some dedifferentiation processes to be reversed and liver specific functions to be restored (Dunn et al., 1991). To support the advantages of 3D culturing, research has shown that sufficient cell-cell junctions are critical in terms of hepatocytes preserving their hepatic phenotype

(Vinken et al., 2006) (Gieseck et al., 2014). Since noticing these phenomena, multiple different 3D cultivation techniques have been introduced to ensure sufficient cell-cell and cell-matrix interactions. These include forced floating method, agitation-based approaches, hanging drop method, utilisation of matrices and scaffolds and utilisation of microfluidic cell culture platforms. Next sections will review the 3D methods themselves and finally the maturational effects observed through their use.

5.2.1 Methods of 3D monoculturing

Forced floating method is based on modifying the vessel surface with a non-adhesive coating so that cell attachment is prevented, resulting in forced-floating cells. This promotes cell-cell contacts and thus multi-cellular sphere formation. (Gupta et al., 2016). Agitation based approaches, instead, include spinner flask bioreactors and rotational culture systems. Cell suspension is placed in a container and kept in motion, either by gentle stirring or rotation of the container. The continuous motion prevents the cells from adhering to the container walls. As a result, they start to form cell-cell interactions. (Gupta et al., 2016). As the third method, in hanging drop method small aliquots of a single cell suspension are pipetted into the wells of a tray. The cell density of the seeding suspension may be altered depending on the required size of spheroids. The tray is then inverted and so the aliquots turn into hanging drops, staying in place due to surface tension. Cells assemble at the tip of the drop at the liquid-air interface. (Breslin & O'driscoll, 2012). Second to last, matrices and scaffolds are designed to mimic ECM and the basement membrane. Matrices often include different basement membrane proteins, such as components for cellular signalling and growth factors, to induce cell differential and growth. Cells may be either embedded and grown within the gel or on top of it. They are allowed to create both cell-cell and cell-ECM interactions as they develop into structures similar to those found in the origin tissue. Prefabricated scaffolds may be also used to aid 3D culture, as cells seeded in them can migrate between fibres and attach to them. 3D structures are formed as the interstitial space between fibres is filled by growing and dividing cells. 3D printing of scaffolds has been widely recognized as a valid technique as well as deriving decellularized ECM scaffolds from e.g. mouse, rat and pig livers. (Breslin & O'driscoll, 2012). Finally, microfluidic cell culture platforms consist of a main microfluidic channel in which there is an array of micropillars. Cell suspension is passed through the micropillars to immobilize the cells within the pillars and support their growth, enabling cell-cell interactions. Afterwards a collagen matrix is passed through the system to form a thin layer over the cells allowing for cell-matrix interactions. (Breslin & O'driscoll, 2012). Table 1, an adapted and modified version of a

table presented by Breslin and O-driscoll, summons some of the advantages and disadvantages related to the different 3D cultivation methods introduced.

Table 1. Advantages and disadvantages of different three-dimensional culture system methods, an adapted and modified version (Breslin & O'driscoll, 2012)

Method type	Advantages	Disadvantages	References
Forced floating	Simple and cheap, thus well qualified for high-throughput testing. Produced spheroids are accessible with only a little effort.	Cell size and shape may vary if not controlled by fixed number of cells per well.	(Ivascu & Kubbies, 2006)
Agitation based approaches	Large-scale production easily attainable as cell culturing is simple. Nutrient transport assisted by motion of culture. Produced spheroids are accessible with little effort.	Requires more specialized equipment. Cells may be exposed to shear force in spinner flasks. Little control on cell number and spheroid size.	(Kelm et al., 2003)
Hanging drop	Homogenous spheroids are well qualified for high-throughput testing and accessible with only a little effort. May be done in an inexpensive manner using 96-well plate.	Medium exchanging may be difficult due to small culture volume. May be more costly if using specialized plates.	(Foty, 2011)
Matrices and scaffolds	May incorporate growth factors. Provide 3D support mimicking *in vivo* setting.	May be high-cost for a large-scale production. Produced spheroids may be difficult to retrieve.	(Lee et al., 2007)
Microfluidic cell culture platforms	Well qualified for high-throughput testing.	May be costly due to specialized equipment.	(Tan et al., 2001)

It has been shown that stem cell-derived hepatocytes profit the most from a dynamic 3D perfusion culture when compared to static cultures in terms of induced maturation and prolonged maintenance of functionality (Sivertsson et al., 2013a). Some groups have already begun combining different techniques with a success, examples being Wang's scaffold and a rotating bioreactor with mouse cells (Y. Wang et al., 2012). However, as stated in *Table 1* above, these techniques rely on

expensive and highly specialized equipment and involve interruptive operations during the differentiation process causing unwanted influence on the process.

5.2.2 Maturational effects of 3D monoculturing

Morphology

While most of the focus on research projects goes to measurable mRNA and activity levels, some groups have included reports on the morphological enhancements in 3D cultivated cells. For example, according to Baharvand and colleagues, 2D cultured cells displayed some key morphological characteristics of hepatocytes including binuclei and polyhedral contours. However, only the cells cultured in 3D migrated out of the aggregates and formed cordlike and multi-layered structures, such as the ones *in vivo*. (Baharvand et al., 2006). A few other study groups found similar results with 3D cultured hPSC-derived hepatocytes inducing improved polarization, microvilli at apical side and bile canaliculi formation as well as demonstrating overall better long-term phenotype when compared to cells in 2D culture, which began forming large vacuoles and detaching from culture surface (Freyer et al., 2016; Gieseck et al., 2014). However, it was also reported that while 3D cultures presented these mature elements, the cell population was quite heterogeneous (Freyer et al., 2016).

Albumin secretion

While the relation of albumin secretion in 2D and 3D cultures varied in different studies, no study concluded that 3D cultures producing less albumin than the conventional 2D ones. For example, Baharvand et al. reported that in their experiments, where embryoid bodies were implanted into collagen scaffolds for 3D and dishes for 2D, no significant difference was found in the albumin secretion (Baharvand et al., 2006). Later on another study came into the same conclusion (Ramasamy et al., 2013). However, multiple other studies have shown 3D cultures to significantly improve albumin secretion, both in causing higher peak values and retaining adequate secretion for longer (Ardalani et al., 2019; Freyer et al., 2016; Leclerc et al., 2017; Mun et al., 2019; Subramanian et al., 2014; Takayama et al., 2013). The secretion rate was found to be mostly 1.5-3 fold greater in 3D compared to 2D culture and even consistent with the level of PHHs in the study conducted by Mun and colleagues (Freyer et al., 2016; Mun et al., 2019; Takayama et al., 2013).

Urea secretion

Urea secretion was mostly found to be improved in 3D cultural setting when compared to 2D (Ardalani et al., 2019; Baharvand et al., 2006; Freyer et al., 2016; Mun et al., 2019; Ramasamy et al., 2013; Takayama et al., 2013). The difference, however, wasn't as significant as with albumin secretion, and one study even reported that the two different cultures produced comparable amounts by their last measuring day (Freyer et al., 2016). Nevertheless, as stated earlier, urea secretion as such is a marker or the function of arginase and thus doesn't reveal that much about the functionality of the whole urea cycle.

Triglycerides and lipoproteins

According to Mun and colleagues, gene expression related to lipid metabolism, including fatty acid uptake and metabolism, triglyceride hydrolysis, beta oxidation and cholesterol metabolism and secretion, were upregulated in 3D compared to 2D differentiated hepatocytes (Mun et al., 2019). Indeed, fatty acyl CoA was found to be significantly increased in the 3D cultures. They also responded well to excess fatty acid treatment and reflected steatosis phenotypes such as lipid droplet accumulation, increased triglycerides, and mitochondrial impairment. (Mun et al., 2019). Accordingly, other study groups have also discovered glycerolipid metabolic process, regulation of lipid transport, regulation of cholesterol transport (for example *apolipoprotein F*) and finally cholesterol and lipid homeostasis to be among the upregulated genes (Leclerc et al., 2017; Ramasamy et al., 2013; Sivertsson et al., 2013b). Consistent with the mRNA findings, Thi and colleagues have reported higher levels of secreted lipoproteins in 3D setting (Thi et al., 2020).

Drug metabolizing enzymes

Each study encountered discovered CYP expression levels and activities to be improved when comparing 3D culturing to 2D (Ardalani et al., 2019; Baharvand et al., 2006; Freyer et al., 2016; Gieseck et al., 2014; Leclerc et al., 2017; Mun et al., 2019; Ramasamy et al., 2013; Sivertsson et al., 2013b; Subramanian et al., 2014; Takayama et al., 2013). Of particular interest, CYP3A4 and CYP7A1 both were found to be upregulated and showed higher activity levels in 3D apart from a study conducted by Freyer and colleagues, where 2D cultures produced insignificantly higher CYP3A4 activities (Freyer et al., 2016). However, despite the improvement in CYP3A4 activity, the activity level is still at most a quarter of what PHHs show (Freyer et al., 2016; Gieseck et al., 2014; Mun et al., 2019). *CYP3A7* expressions, which is thought to be related to immaturity, were found to be decreased by even 140-fold (Gieseck et al., 2014).

Other genetic components

As stated earlier, AFP is generally used as a marker of immaturity of hepatocytes. Different studies have reported conflicting results as Gieseck's, Leclerc's, Ardalani's and Freyer's research groups reported on significantly, even 20-fold lower transcript amounts in 3D cultures when compared to 2D (Ardalani et al., 2019; Freyer et al., 2016; Gieseck et al., 2014; Leclerc et al., 2017) while Subramanian's and Baharvand's groups found the situation to be the opposite (Baharvand et al., 2006; Subramanian et al., 2014). Protein levels for *ASGPR1* and transcript levels for $HNF4\alpha$ were found to be increased in 3D culture, while at least for $HNF4\alpha$ they were found to be far behind mature hepatocyte levels (Ardalani et al., 2019; Freyer et al., 2016; Subramanian et al., 2014).

5.3 3D co-cultures of hPSC-hepatocytes with non-parenchymal cells

The next step on improving the maturity of hPSC-derived hepatocytes has been to combine the two techniques presented and reviewed above, namely co-culturing and 3D culturing.

5.3.1 Methods of 3D co-culturing

Once again there are multiple different techniques which may be used to achieve a 3D co-culture. Some are applications of the previously presented techniques, such as stacking of cell sheets. For the past few years increasing interest has been shown towards generation of 3D organoids due to their improved state of maturity.

Organoids

Organoids are often defined as *in vitro* 3D self-aggregated and self-organized, progenitor-cell derived cell configurations used as organ models. These progenitor cells, such as iPS-cells, have been capable of forming an organ-like arrangement and exhibiting some crucial functions of the organ with minimal external cues. While some organoids are developed with the help of another cell type along hPSCs, most of the studies included in this literature review utilized co-differentiation into several different cell types, which then together formed organoids.
As one might guess, organoid generation requires a lot of careful work and planning. For example, intestinal organoids are generated with the help of exactly measured concentrations of growth factors present in organogenesis processes (Yip et al., 2018). In addition, other important aspects to consider include the number and density of the cells used, their culture conditions including the presence/absence of extracellular matrix and finally the most suitable engineered geometry to

promote the aggregation and differentiation processes (Brassard & Lutolf, 2019). Some 3D approaches include bioengineered matrices and synthetic scaffolds while some require less handwork. Figure 2 (JP & TG, 2020) summons some of the current strategies for generation of hPSC-derived organoids.

Already a decade ago a study showed embryonic stem-cell derived hepatocytes in an organoid presenting a level of functionality comparable to or even better than that of primary mouse hepatocytes (Amimoto et al., 2011). After that, other studies have also come to the conclusion of heterotypic cell-cell interactions being critical for self-organization and paracrine signals secreted by non-parenchymal cells being important for hepatic maturation (Asai et al., 2017; Camp et al., 2017). Thus, the technique has become more and more popular especially during the last couple of years while bringing considerable progress to the maturity of the acquired hPSC-derived hepatocytes.

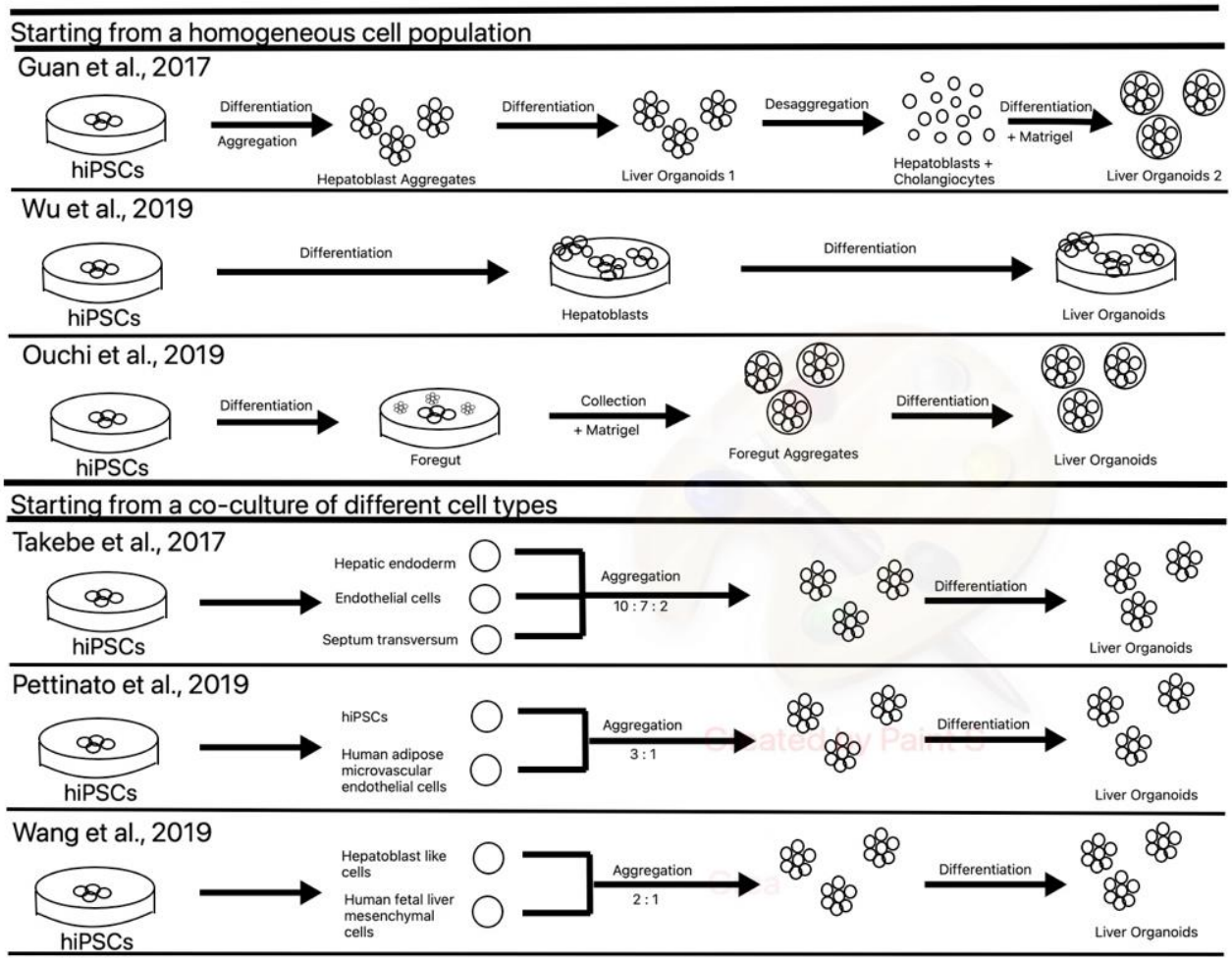

Figure 2. Some of the current strategies for generation of human pluripotent stem cell-derived liver organoids. An adapted and modified version (JP & TG, 2020).

5.3.2 Maturational effects of 3D co-culturing

Morphology

All studies found presented pictural evidence or description on independently formed organoid units (Ardalani et al., 2019; Guan et al., 2017; Ouchi et al., 2019; Pettinato et al., 2019; Takebe et al., 2017; S. Wang et al., 2019; Wu et al., 2019). Wang and colleagues stated that morphologically their organoids resembled human adult liver-derived hepatic organoids (S. Wang et al., 2019). Moreover, Ardalani et al. reported their organoids being organized so, that the co-cultured endothelial cells formed capillary-like structures evidenced by CD31 staining (Ardalani et al., 2019).

Albumin secretion

As expected, due to different organoid strategies, studies reported on rather varying levels of albumin secretion when compared to one another. Guan and colleagues found their organoids consisting of only iPSCs, which had differentiated into cholangiocytes and hepatocytes, to produce albumin mRNA levels comparable to those in primary human hepatocytes (PHHs) in liver tissue (Guan et al., 2017). Even better results were announced by Takebe et al., who stated that their organoids with umbilical cord vein endothelial cells and bone marrow mesenchymal cells presented higher levels of actual albumin production than PHHs (Takebe et al., 2017). However, Wu and their research group with organoids consisting of only iPSCs, which had differentiated into cholangiocytes and hepatocytes, reported on the level of albumin secretion on day 35 being comparable to that of HepG2 and fetal liver cells but only about a third of that of cryopreserved PHHs (Wu et al., 2019). Furthermore, Pettinato et al. and Wang et al. with their co-cultures with endothelial and mesenchymal cells, respectively, came to the conclusion that their organoids secreted albumin at lower levels than even PHHs (Pettinato et al., 2019; S. Wang et al., 2019).

When compared to monocultures, it was clear that organoids were able to produce albumin at higher rates. Pettinato's research group found albumin to be secreted at statistically significant 1.65-fold levels in 3D co-culture than 3D monoculture (Pettinato et al., 2019). Ardalani et al., on the other hand, found their organoids with non-parenchymal endothelial cells to produce albumin at statistically significant 2 – 50-fold levels when compared to aggregate monocultures. For example the addition of cholangiocyte cells to the co-culture led to a significant decrease in the production levels. (Ardalani et al., 2019).

Urea secretion

The results for urea secretion on different study reports seemed to be more in line with one another when compared to albumin production. Pettinato's and Wang's research groups reported that their organoids, with endothelial cells and mesenchymal cells in respective order, produced significantly lower levels of urea than PHHs (Pettinato et al., 2019; S. Wang et al., 2019). Wu et al. reported that organoids, consisting of only iPSC-derived cholangiocytes and hepatocytes, produced urea at day 35 at comparable levels to HepG2 but only about half that produced by cryopreserved PHHs (Wu et al., 2019).

Pettinato et al. showed that hepatocyte monoculture produced a significantly higher amount of urea than organoids, which could be explained by the difference in cell seeding density ratio between the two conditions (Pettinato et al., 2019). Ardalani et co. reported on urea being secreted at significantly higher 2 – 50-fold levels in organoids with hepatocytes and endothelial cells when compared to monoculture. Once again, a decrease in the produced urea levels was noted when cholangiocyte cells or hepatic stellate cells were added. (Ardalani et al., 2019).

Triglycerides and lipoproteins

Unfortunately, only a few studies reported on their experiments on organoids' triglyceride and lipoprotein related functions and these reports were quite vague. According to Ouchi and colleagues, organoids consisting of hPSC-derived hepatocytes and supportive lineages such as Kuppfer-like cells, expressed highly comparable gene signatures to PHHs, containing major genes associated with lipid metabolism. However, the functional activity in this study was left undetermined. (Ouchi et al., 2019) Pettinato et al. compared the main triglyceride and lipoprotein functions of their endothelial cell co-culture organoids to monoculture and to PHHs. They stated that the results for co- and monoculture displayed equivalent function to PHHs with respect to cytoplasmic accumulation of neural triglycerides and lipids as well as acetylated low-density lipoprotein uptake. (Pettinato et al., 2019) Wu et co. bluntly stated that the hepatic cells in their organoids, consisting of iPSC differentiated into cholangiocytes and hepatocytes, were able to store glycogen and accumulate fatty droplets indicating functioning lipid metabolism (Wu et al., 2019).

Drug metabolizing enzymes

When compared to PHHs and liver tissue, organoids with comparable levels of at least one CYP enzyme have been created. Most of the studies found comparable or even higher CYP450 activity for CYP3A4 when compared to PHHs. (Ouchi et al., 2019; Pettinato et al., 2019; Wu et al., 2019).

Furthermore, Wu and colleagues reported their organoids' CYP3A4 activity at day 45 to be over 80% of that of cryopreserved PHHs (Wu et al., 2019). However, Wang's research group published contradicting results showing significantly lower CYP3A4 activity in organoids than in PHHs (S. Wang et al., 2019). In addition to *CYP3A4*, at least the genes *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP2D6* and *CYP2E1* were shown to be expressed at levels at least comparable to fetal liver, but for now it seems like they don't translate into improved activity levels (Pettinato et al., 2019; Wu et al., 2019).

When compared to monoculture aggregates, the benefit of culturing organoids seems apparent. Following induction with omeprazole, rifampicin and phenobarbital, at least CYPs 1A1, 1A2, 3A4, 3A7, 2B6 and 2C9 displayed significant increases in their activities in organoids compared to monoculture (Pettinato et al., 2019). Indeed, Ardalani et co. reported on their organoids presenting 1.7-fold, 3.1-fold and 1.6-fold higher activity levels for CYP3A4, CYP1A2 and CYP2C9, in respective order, when compared to monoculture aggregates (Ardalani et al., 2019).

Other genetic components

In general it seems like organoids express a vast majority of the most important hepatocyte-enriched genes with similar levels to PHHs (Asai et al., 2017; Takebe et al., 2017; Wu et al., 2019; Zabulica et al., 2019). Along with the already mentioned ones related to albumin and urea secretion, $HNF4\alpha$ was found to be expressed and produced in the range of adult liver (Asai et al., 2017; Takebe et al., 2017; Wu et al., 2019; Zabulica et al., 2019). Along with it, single studies made observations on the levels of inter alia *A1AT*, *TTR*, *HNF1a*, *HNF1b, HNF3a*, *HNF3b*, *HNF4a*, the aromatic hydrocarbon receptor (*AHR*), and the glucocorticoid receptor (*GR*) mRNA levels being at least comparable to those of PHHs (Asai et al., 2017; Zabulica et al., 2019). According to a global transcriptome analysis of liver organoids, their maturity was comparable to PHHs (Takebe et al., 2017).

Unsurprisingly, HNF4α was found to be expressed at significantly higher levels in organoids versus 3D monocultures (Guan et al., 2017; Pettinato et al., 2019). Pettinato and colleagues also reported AFP being secreted by organoids only 85% of the amount secreted by the 3D monocultures indicating better maturity state (Pettinato et al., 2019).

6 CONCLUSIONS AND DISCUSSION

When comparing the chosen measures of maturity presented by 2D co-cultures, 3D monocultures and 3D co-cultures, the last mentioned offered the most convincing and thus most mature results. 3D co-culture was the only culturing method to provide capillary-like structures, albumin production even better than PHHs *in vivo*, highly comparable gene signatures for triglyceride & lipoproprotein metabolism to PHHs and HNF4α transcript and protein levels in the range of PHHs. More interesting point of view would be to compare the increased maturity brought by either 2D co-culturing or 3D monoculturing the hiPSCs. Both methods seemed to improve albumin and urea secretion similarly (levels comparable to PHHs *in vitro*), but according to the studies included in this review 3D monoculture had a greater positive impact on at least CYP enzyme expressions, activities and morphology. However, only few 2D co-culture studies had included measurements for anything other than albumin and urea secretion, and thus it's impossible to confidently deduce which method is able to mature hPSCs better.

There are many complicating factors that should be considered before drawing any conclusions from the results presented in this literature review. Firstly, there are multiple other things affecting the maturity of the hPSCs than only whether they are cultivated in a 2D or 3D environment and whether other type of cells are cultured in close proximity with them. As presented earlier in this review, there are numerous ways of conducting a 3D environment and co-culturing with different culture media and different cell types. Unfortunately, enough studies were not found to deduce anything related to the more precise cultivation methods. One study made a point on liver sinusoidal endothelial cells and hiPS-derived endothelial cells being the best type of cells to co-culture with, while cholangiocytes and hepatic stellate cells brought the worst results (Ardalani et al., 2019). However, since most studies did not test out different combinations with the same study setting, it is hard to conclude anything about the best or worst cell types to co-culture with.

To name a few other difficulties in comparing the study results, apart from albumin and urea secretion, the studies used quite varying parameters for the other measures of maturity. A good example of this would be triglyceride and lipoprotein metabolism, where some studies reported on the ability to accumulate fatty droplets, others on cytoplasmic accumulation. In addition, quite often the research groups had only measured the transcript levels for the genes, which doesn't strictly correlate with the actual protein levels and their activities. Lastly, but perhaps the most importantly, there were quite few studies that were suitable to be included in this review, and thus the probability for random errors is significant.

Naturally, a wish for future would be to do more research on this area. To be able to minimize the amount and impact of confounding factors it's imperative to use the same controlled study settings and cell types. While organoids are clearly the best culture method for more mature results, it would be interesting and benefitting for research to have more studies on the effect that different cell types have on the maturity of hPSC-derived hepatocytes. This way research would be one step closer to determining the underlying mechanisms for the maturation process.

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