Improvement of Human iPS Cell-Derived Hepatocyte Functionality Using 3D Culture System

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Introduction

Human primary hepatocytes are utilized for high-throughput screening in early-stage drug discovery in order to evaluate thousands of potential compounds. Yet, human primary hepatocytes have the disadvantage of a limited supply from a single donor as well as high donor-to-donor variability. To overcome these obstacles, functional human induced pluripotent stem (iPS) cell-derived hepatocytes are highly desirable, as they are available in unlimited quantities from the same donor. However, immaturity and donor-to-donor variability are common drawbacks of iPS cell-derived hepatocytes.

To address hepatocyte maturation, we evaluated multiple methods using 3D cultivation for maturing iPS cell-derived hepatocytes. We compared different 3D culture systems with traditional 2D cultures by analyzing the expression levels of specific cytochrome P450 (CYP) enzymes that play an important role in drug-metabolism. We believed that 3D culture is able to provide a micro-environment that promotes maturation of human iPS cell-derived hepatocytes, potentially facilitating the creation of a human iPS cell-derived hepatocyte panel, which will enable assessment of donor-to-donor variability in iPS cell-derived hepatocyte function.

Materials and Methods

ReproHepato type I™ kit (Cat. No. RCESDH001)

- Cells 1 vial (8.25 million cells/vial)
- Thawing Medium 1 bottle
- Maintenance medium 1 bottle
- Assay Medium 1 bottle
- Supplements

3D culture

- Puramatrix® (3D Matrix Inc.)
- Nanoshuttle™ PL (n3D Bio Inc.)
- Low attachment plate (sumitomo bakelite)
- Bioreactor (Able-Biott 30 mL)

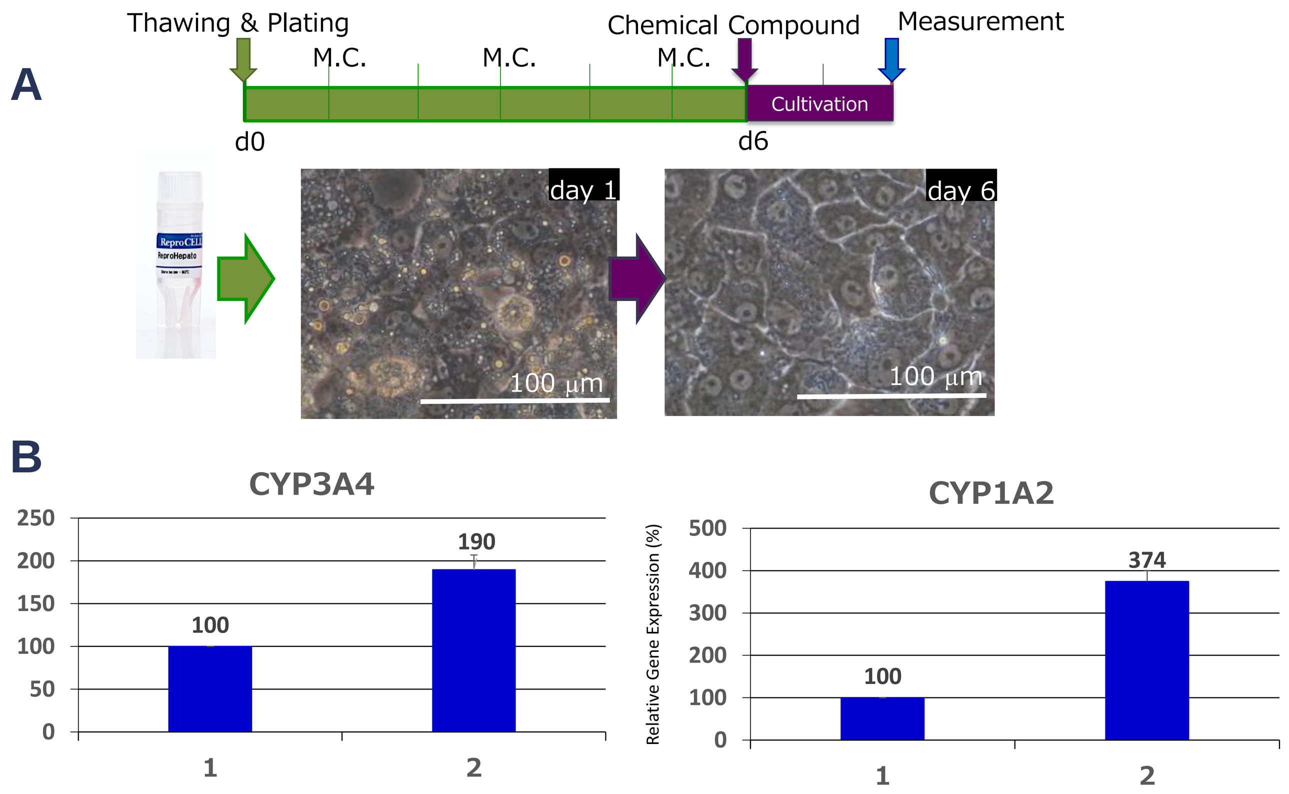
RT-PCR

- CYP3A4 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs00604506_m1)
- CYP1A2 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs00167927_m1)
- CYP2B6 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs04183483_g1)
- CYP2C9 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs02383631_s1)
- CYP2C19 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs00426380_m1)
- CYP2E1 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs00559368_m1)
- CYP2A6 TaqMan Gene Expression Assays (Life technology, Cat.No. Hs00868409_m1)
- GAPDH TaqMan Gene Expression Assays (Life technology, Cat.No. Hs02758991gm1)

CYP3A4 induction assay

- Rifampicin (Sigma, Cat.No. R7382)
- Omeprazole (Sigma, Cat.No. 104)
- Sodium Butyrate NA (Sigma, Cat.No. 303410)
- P450-Glo™ CYP3A4 Assay with Luciferin-IPA (Promega, Cat. No. V9002)

Characterization of ReproHepato



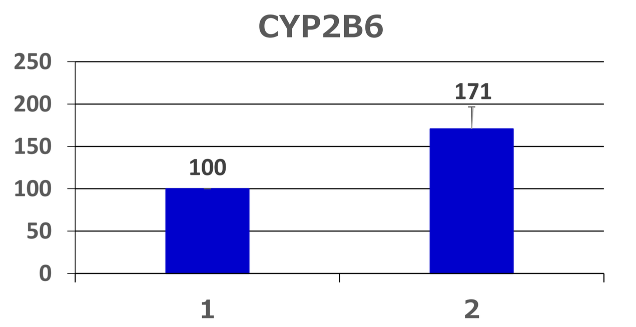


FIGURE 1: ReproHepato for CYP P450 activity/induction Assay.
FIGURE 1A: ReproHepato was thawed and seeded onto Corning® Matrigel® coated 24 well-plate.
FIGURE 1B: The induction of CYP3A4, 1A2, and 2D6 (average of 3 different lots). The average basal level of ReproHepato was taken as 100%. The numbers on the graph represent relative expression after normalization. The error bar represent the standard deviation.

3D cultivation of ReproHepato ①

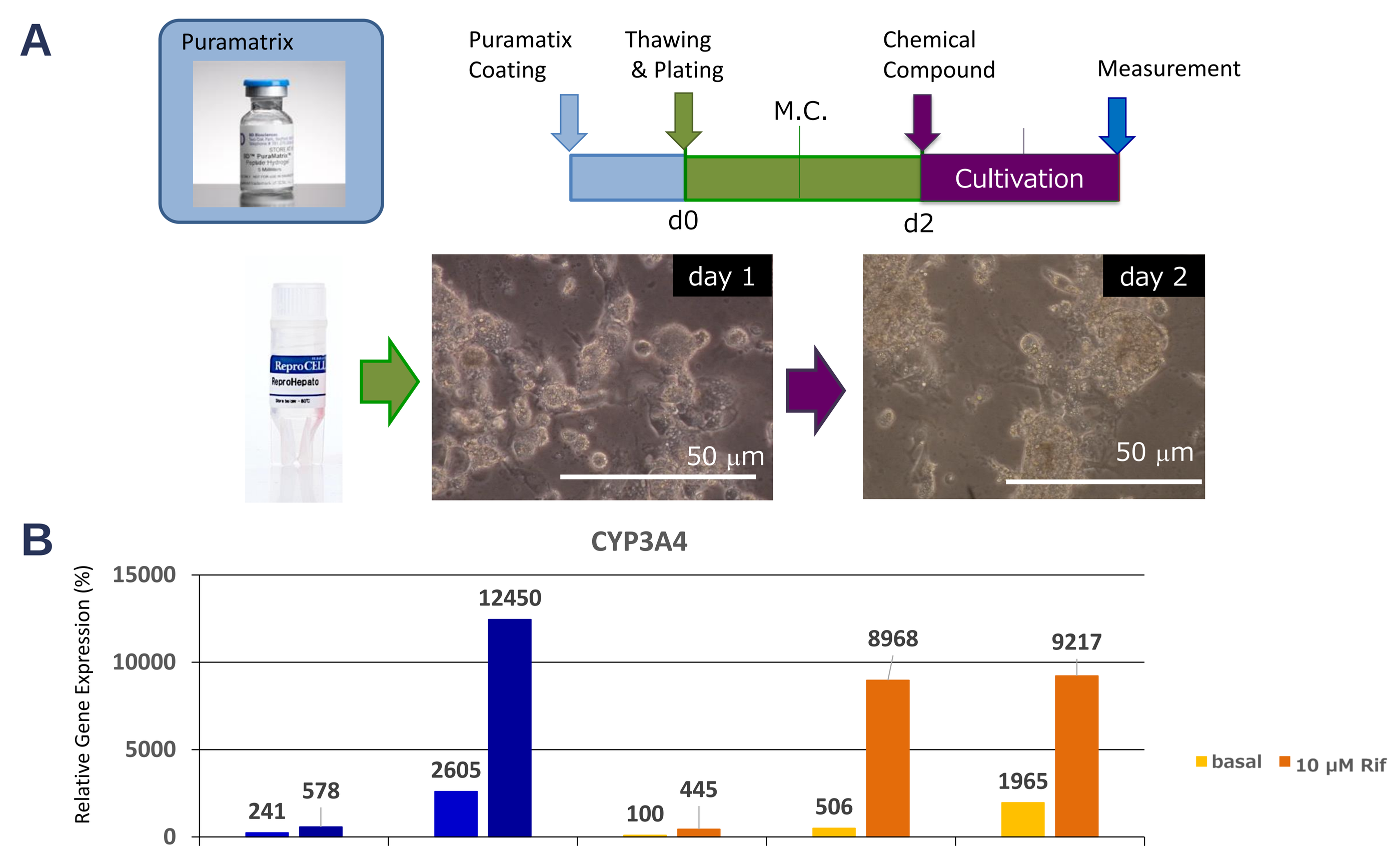


FIGURE 2: 3D cultivation of ReproHepato Puramatrix™.
FIGURE 2A: The scheme of thawing and assay of ReproHepato Type1 using Puramatrix.
FIGURE 2B: The basal level and induction of ReproHepato on Puramatrix (blue, 2) was also better than those of primary hepatocyte (yellow, 3, 4 & 5). The number on the graph show relative expression after normalization. This data has supported the fact that 3D cultivation could greatly improve the maturation of iPS cell-derived hepatocytes.

3D cultivation of ReproHepato ②

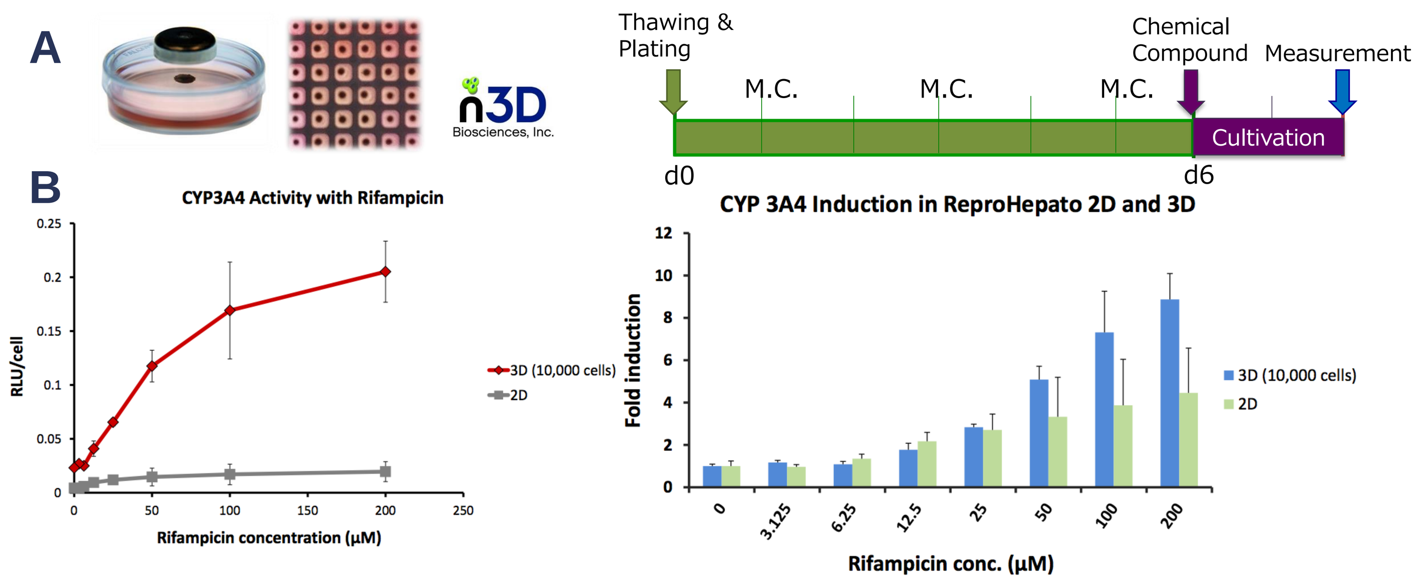


FIGURE 3: hiPSC-hepatocyte spheroids demonstrate superior CYP3A4 activity and inducibility over 2D monolayer at day 9 of culture.
FIGURE 3A ReproHepato hepatocytes were thawed and immediately plated in both 2D monolayer (75,000 cells, 96-well format) and printed into 3D magnetized spheroids (10,000 cells/spheroid, 384-well format) after 2 hrs incubation with Nanoshuttle PL. Hepatocytes were cultured for 6 days before exposure to rifampin (0-200 um) for 72 hours. At day 9, CYP3A4 activity was measured using the p450-Glo assay.
FIGURE 3B CYP3A4 activity of ReproHepato spheroids is induced by in a dose-dependent manner, as similar kinetics with primary hepatocytes.

Hepatic differentiation by 3D culture

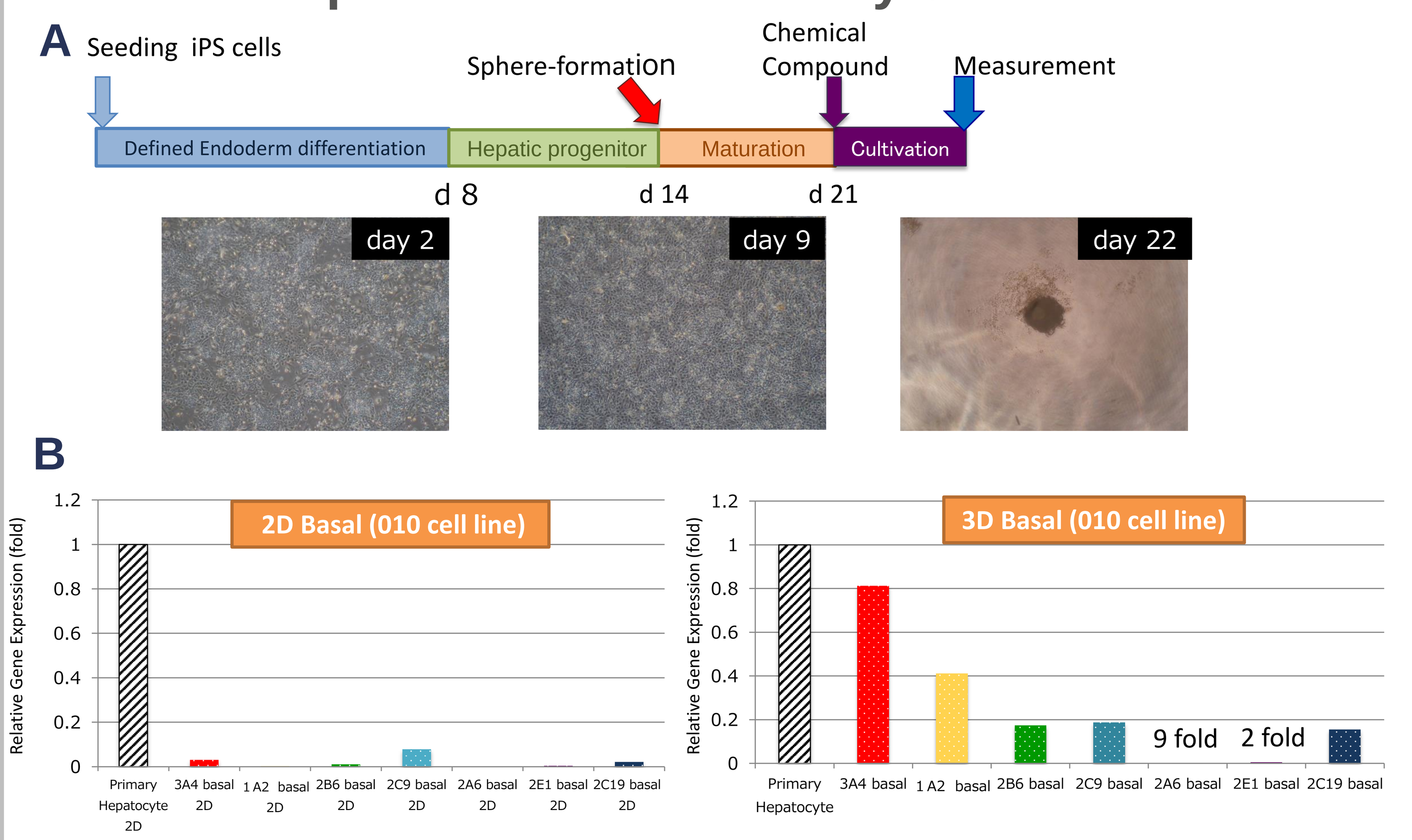


FIGURE 4: 3D cultivation could greatly improve the maturation of iPS cell-derived hepatocytes.
FIGURE 4A Schematic diagram representing hepatic differentiation of human iPS cells and their morphology.
FIGURE 4B The basal level of CYP families between 2D- (left) and 3D-differentiation (right). In 2D-differentiated hepatocytes, the overall expression was low compare to primary hepatocyte. In 3D-differentiated hepatocytes. CYP3A4, 1A2 and 2B6 has greatly improved by comparing with those of 2D-differentiated hepatocyte. CYP2C9 and 2C19 were both increased as well, while CYP2A6 and 2E1 still have low basal level. The CYP basal level of primary hepatocyte was taken as 1 and normalizes the expression of other samples. The number on the graph show relative expression after normalization.

Optimization of Hepatic Differentiation: ❶2D Preliminary Study

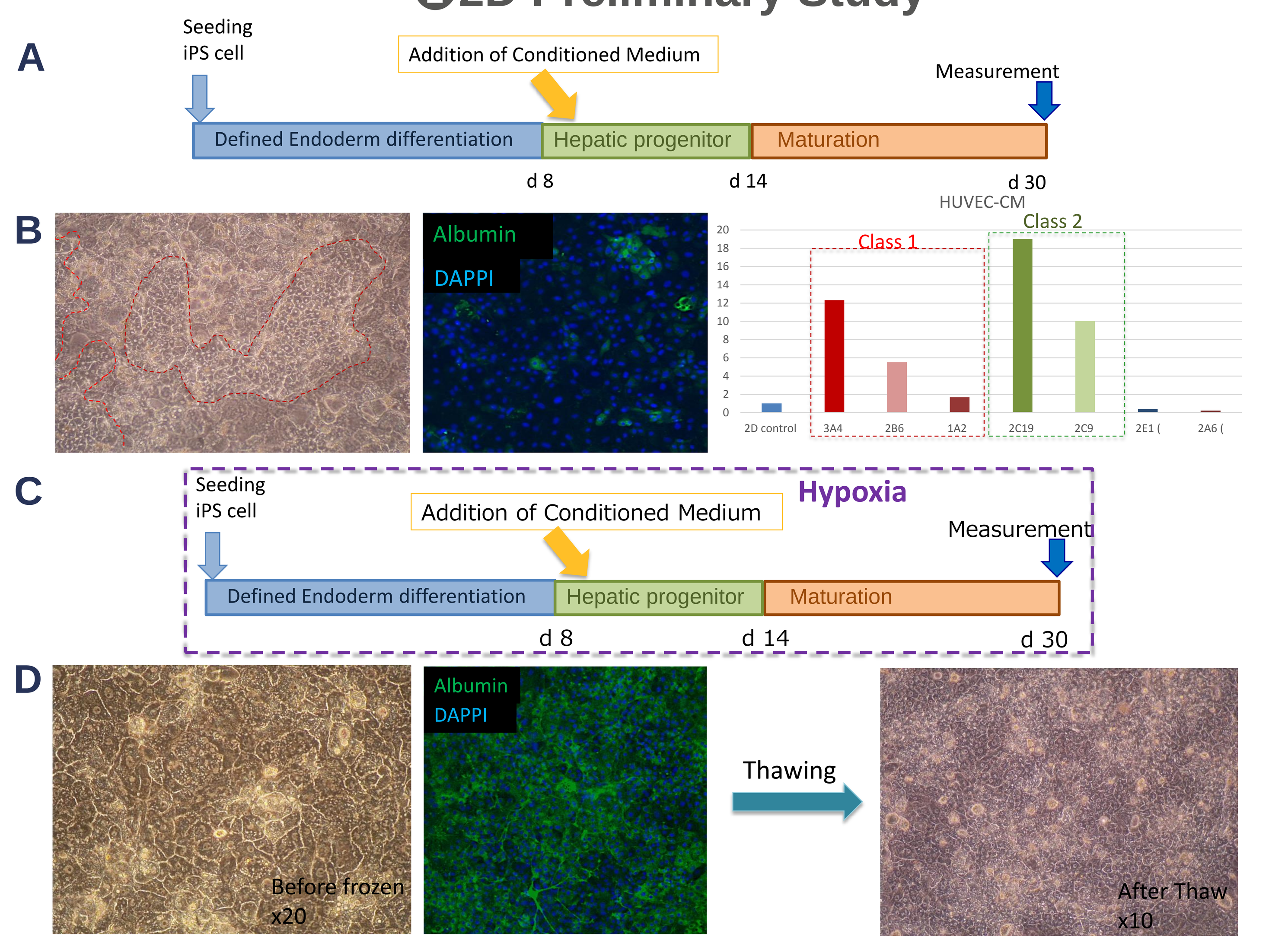


FIGURE 5: Conditioned Medium & Hypoxia could improve the maturation of iPS cell-derived hepatocytes.
FIGURE 5A Schematic diagram representing hepatic differentiation of human iPS cells with Conditioned Medium.
FIGURE 5B The morphology of Conditioned Medium-treated differentiated hepatocytes. The red outline represents areas of good morphology. The red-circled patch with good morphology was positive for human albumin antibody. The basal level of CYP class 1 (Red) and 2 (Green) were increased compare to those of ReproHepato (Blue).
FIGURE 5C Schematic diagram representing hepatic differentiation of human iPS cells with Conditioned Medium under hypoxic conditions.
FIGURE 5D The good morphology of hepatocytes was uniformly seen all over, and the albumin expression was uniform. The good morphology remained unchanged after the frozen hepatocytes were thawed.

❷Application of Preliminary Study to 3D Culture

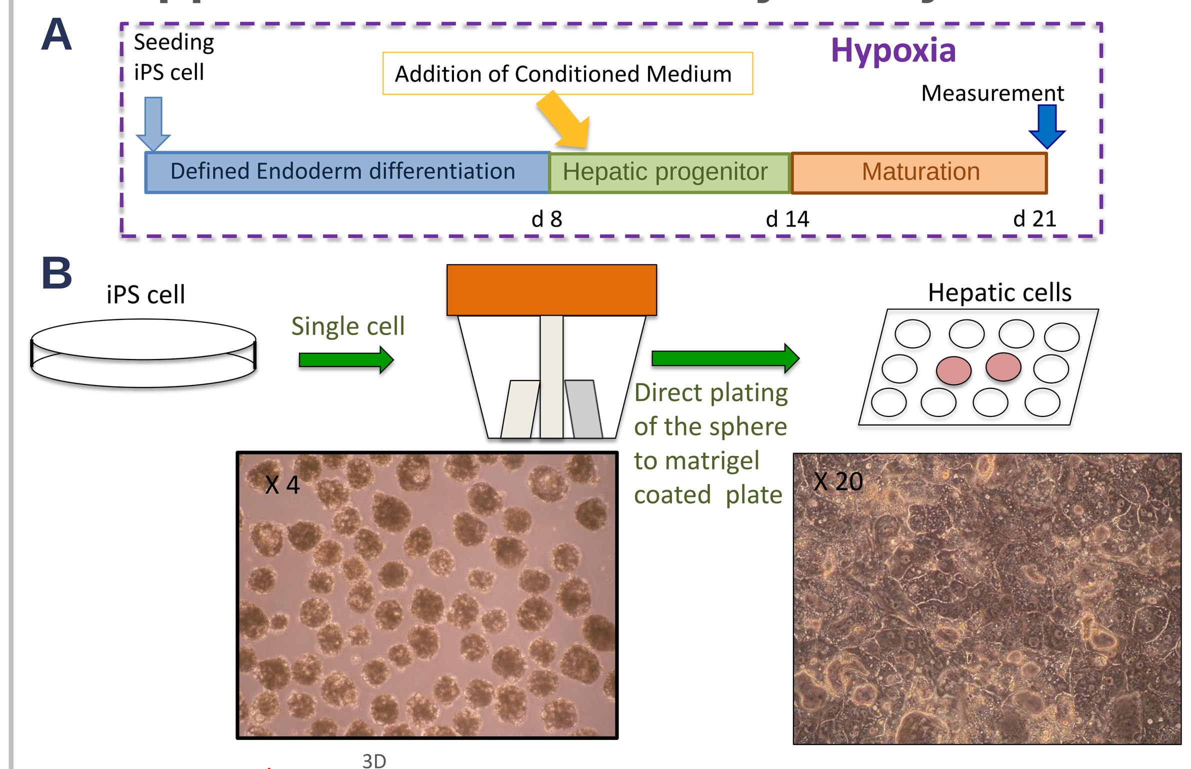


FIGURE 6: Conditioned Medium & Hypoxia in 3D cultivation should improve the maturation of iPS cell-derived hepatocytes.
FIGURE 6A Schematic diagram representing hepatic differentiation of human iPS cells with Conditioned Medium under hypoxic conditions.
FIGURE 6B Schematic diagram representing 3D hepatic differentiation of human iPS cells, morphology of hepatic sphere, and morphology of hepatocyte after attachment.

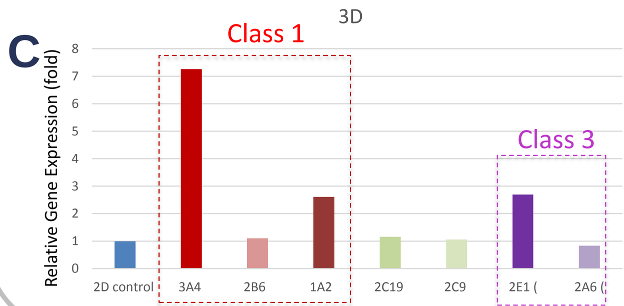


FIGURE 6C The basal levels of CYP class 1 (Red) and 3 (Purple) expression were increased compared to those of ReproHepato(Blue). However class 2 (Green) was not much affected by 3D cultivation.

Conclusion

Simplified FDA guidelines

Category	CYP family	Basal expression		Induction	
1	CYP3A4	similar to primary hepatocyte		at least 5 fold	
		2D☑	3D☑	2D△	3D☑
	CYP1A2	similar to primary hepatocyte		at least 5 fold	
		2D△	3D☑	2D△	3D☐
	CYP2B6	similar to primary hepatocyte		at least 3 fold	
		2D△	3D☑	2D△	3D☐
2	CYP2C9	similar to primary hepatocyte			
		2D△	3D☑		
	CYP2C19	similar to primary hepatocyte			
		2D△	3D☑		
3	CYP2D6	similar to primary hepatocyte			
		2D☐	3D☐		
	CYP2E1	similar to primary hepatocyte			
		2D☒	3D△		
	CYP2A6	similar to primary hepatocyte			
		2D☒	3D△		

- Cultivation of ReproHepato Type1 (Cat. No. RCESDH001) in 3D culture increases both basal level and induced CYP3A4 expression to a greater extent than primary hepatocytes.
- 3D spheroid-formation during hepatic differentiation to improves the basal expression level of all 3 class expression further.

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