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Laminin-511 and laminin-521-based matrices for efficient hepatic specification of human pluripotent stem cells

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ABSTRACT

Human pluripotent stem cells (hPSCs) have gained a solid foothold in basic research and drug industry as they can be used *in vitro* to study human development and have potential to offer limitless supply of various somatic cell types needed in drug development. Although the hepatic differentiation of hPSCs has been extensively studied, only a little attention has been paid to the role of the extracellular matrix. In this study we used laminin-511, laminin-521, and fibronectin, found in human liver progenitor cells, as culture matrices for hPSC-derived definitive endoderm cells. We observed that laminin-511 and laminin-521 either alone or in combination support the hepatic specification and that fibronectin is not a vital matrix protein for the hPSC-derived definitive endoderm cells. The expression of the laminin-511/521-specific integrins increased during the definitive endoderm induction and hepatic specification. The hepatic cells differentiated on laminin matrices showed the upregulation of liver-specific markers both at mRNA and protein levels, secreted human albumin, stored glycogen, and exhibited cytochrome P450 enzyme activity and inducibility. Altogether, we found that laminin-511 and laminin-521 can be used as stage-specific matrices to guide the hepatic specification of hPSC-derived definitive endoderm cells.

1. Introduction

Hepatocytes in the liver are in a continuous interaction not only with their neighboring cells but also with an extracellular matrix (ECM). The ECM provides essential cues for cell proliferation, migration, and differentiation [1]. The secretion of the ECM starts already at the embryonic stage, and its remodeling is an important mechanism by which tissue formation is regulated [2,3]. Indeed, the topology and composition of the ECM are not static during embryonic development or in tissue homeostasis and regeneration. Moreover, the ECM chemistry varies between the zones in the liver [4]. However, the role of the ECM in specific lineage stages is largely undiscovered. Human pluripotent stem cells (hPSCs) offer a useful *in vitro* tool to study the cell-ECM interactions during human development as the *in vivo* research on human development is typically hindered by ethical concerns. In addition, studying specific ECM proteins can lead to the development of new bioinspired matrices for *in vitro* hepatic differentiation.

In vitro hepatic differentiation of both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) has been tremendously studied [5–10]. The hepatic differentiation is most often guided through definite endoderm (DE) and hepatic progenitor to obtain hepatocyte-like cells [11] as this step-wise

Abbreviations: ACM, acellular matrix; Act A, Activin A; AAT, alpha-1 antitrypsin; AFP, alpha fetoprotein; ALB, albumin; AhR, aryl hydrocarbon receptor; BMP, bone morphogenetic protein; CK, cytokeratin; CXCR-4, chemokine receptor type 4; CYP, cytochrome P450; DE, definite endoderm; DEX, dexamethasone; ECM, extracellular matrix; F-actin, filamentous actin; FBS, fetal bovine serum; FGF, fibroblast growth factor; HCM, hepatocyte culture medium; hESC, human embryonic stem cell; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; hPSC, human pluripotent stem cell; hiPSC, human induced pluripotent stem cell; LN, laminin; NR1I2, nuclear receptor subfamily 1, group I, member 2; NR3C1, nuclear receptor subfamily 3, group C, member 1; OCT4, octamer-binding transcription factor 4; OMZ, omeprazole; OSM, oncostatin M; PHH, primary human hepatocyte; qPCR, quantitative polymerase chain reaction; rhEGF, recombinant human epidermal growth factor; SSEA, stage-specific embryonic antigen; Wnt, wingless type.

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procedure follows the natural differentiation process [12]. In the majority of differentiation protocols cell-matrix interactions have been neglected; differentiation process is guided solely by soluble factors, and hPSC derivatives are cultured on the same matrix used initially for plating stem cells. For creating a robust and scalable stem cell differentiation protocol cell culture matrix should desirably be well-defined and xeno-free. However, the optimal culture matrix for *in vitro* hepatic differentiation of the DE cells is poorly understood. A purpose-driven development of new culture matrices should begin by learning from the composition and distribution of the ECM proteins in the tissue of interest. It has earlier been suggested that new biomaterials for liver cell cultures can be found by characterizing ECM proteins in a liver acellular matrix (ACM) [13]. We recently proposed a simpler approach to find lineage stage-specific matrices by using cell lines [14]. We created an ACM from human liver progenitor HepaRG cells to mimic the matrix of liver progenitors and used the ACM for differentiating hPSC-derived DE cells according to our hypothesis that liver progenitor-like matrix could induce efficient hepatic lineage specification of DE cells. Indeed, we were able to show that liver progenitor-like matrix supported the differentiation of DE cells towards hepatocytes.

To produce a chemically defined matrix that mimics the HepaRG-ACM, in this study, we characterized the matrix components produced by HepaRG cells and found that laminin-511 (LN-511), laminin-521 (LN-521), and fibronectin were highly expressed. Furthermore, we showed that LN-511 and LN-521 can be used as culture matrices for hepatic specification and differentiation of hPSC-derived DE cells.

2. Materials and methods

2.1. Characterization of the ECM proteins in liver progenitor cells

HepaRG cells [15] obtained from Biopredict (Saint-Grégoire, France) were cultured for two weeks in earlier described culture conditions. The ECM mRNA and protein expression of the HepaRG cells was characterized by conventional RT-PCR and immunofluorescence, respectively (see sections 2.4–2.6 below).

2.2. Cell cultures

The hESC lines WA07 and H9 [16] and hiPSC line iPS(IMR90)-4 [17] were bought from WiCell research institute. H9 cells were genetically modified to H9-GFP cells as earlier reported by us [18]. All the stem cell lines were maintained in Matrigel culture system (BD Biosciences, 356230, 0.5 mg per one 6-well plate) in mTeSR™1 medium (STEMCELL™ Technologies, 05850) which was renewed daily. The WA07 and iPS(IMR90)-4 cells were passaged at ratios of 1:4–1:8 by using Versene 1:5000 (Invitrogen, 15040033) and the H9-GFP cells were passaged at ratios of 1:4–1:6 by using Dispase (STEMCELL™ Technologies, 07923). The WA07, H9-GFP, and iPS(IMR90)-4 cells used in this study were at passages p40, p25(20)-p26(21), and p18 + 40(15)-p18 + 42(17), respectively.

HepaRG cells were used as control cells in real-time qPCR. The HepaRG cells were cultured for two or four weeks in the conditions described previously [15,19]. In the four-week culture, the medium was supplemented with 2% DMSO during the last two weeks to induce the hepatic maturation. All the cell cultures were maintained at 37 °C in a humid atmosphere with 5% CO_2.

2.3. Differentiation of hPSCs to DE and hepatic cells

The iPS(IMR90)-4, WA07, and H9-GFP cells were induced to DE cells during six days as reported earlier [14]. RPMI-1640 medium (Gibco, 31870-025) with earlier described supplements [20,21] 1 × B-27 (Gibco, 17504-044), 100 ng/ml Activin A (PeproTech, 120-14E), 75 ng/ml Wnt-3a (R&D Systems, 5036-WN), and 1 mM (day 0) - 0.5 mM (days 1–5) sodium butyrate (Sigma Aldrich, B5887) was used for the H9-GFP cells. For the iPS(IMR90)-4 and WA07 cells the media were supplemented with 1 × B-27 and 100 ng/ml Activin A.

For hepatic specification we plated the DE cells on seven different matrices with all possible combinations of LN-521, LN-511, and fibronectin (M1-M7; Fig. 2B). Human rLN-511 and human rLN-521 (Biolamina; LN511-01 and LN521-01, respectively) were used at a concentration of 20 μg/ml and fibronectin (Sigma-Aldrich, F0895) at a concentration of 25 μg/ml. All the coating solutions were prepared in 1 × DPBS with calcium and magnesium and incubated in culture wells either for two hours at 37 °C (fast coating) or for overnight at 4 °C (slow coating). After the use, coating solutions were collected, stored at −20 °C, and reused up to two times. Standard tissue culture treated dish was used as a control. The DE cells were gently detached by an enzyme-free Cell Dissociation Buffer (Gibco, 13151-014) for 15 min at 37 °C followed by an Accutase cell detachment solution (Millipore, SCR005) for one to two minutes at room temperature. The H9-GFP-derived DE cells were detached from 6-well plates and seeded at a ratio of 1:1 onto the matrices. To find the optimal seeding protocol for each cell line, the WA07 and iPS(IMR90)-4-derived DE cells were first seeded to M1 at the following densities: 20 000 cells/cm^2, 50 000 cells/cm^2, and 100 000 cells/cm^2. Later the differentiation was performed with the optimal density of 50 000 cells/cm^2 for WA07 and 100 000 cells/cm^2 for iPS(IMR90)-4 cells.

The DE cells on all the studied matrices were cultured in Hepatocyte Culture Medium (HCM™ SingleQuots™ Kit; Lonza CC-4182, without rhEGF and gentamicin-amphotericin-1000) supplemented with 5 ng/ml fibroblast growth factor 4 (FGF4, PeproTech, 100-31), 10 ng/ml bone morphogenetic protein 2 (BMP2, PeproTech, 120-02), and 10 ng/ml BMP4 (PeproTech, 120-05) for four days. During the following four to six days the cells were incubated in HCM supplemented with 10 ng/ml hepatocyte growth factor (HGF, PeproTech, 100-39), 10 ng/ml Oncostatin M (OSM, PeproTech, 300-10T), and 0.1 μM Dexamethasone (DEX, Sigma-Aldrich, D4902). In the last step of differentiation the cells were cultured in HCM supplemented with 0.1 μM DEX and 10 ng/ml OSM or only with DEX for four to six days.

2.4. Immunofluorescence staining

For immunofluorescence staining cells were cultured in either 8-well Lab-Tek® Chamber Slide™ systems (Nunc, 177445) or black 96-well μ-plates (ibidi, 89626). The cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with either 0.1% Triton X-100 or 0.5% Saponin for 10 min except for CXCR-4 staining, and blocked with 10% normal goat or donkey serum (Millipore) for one hour. The cells were then incubated overnight at 4 °C with the primary antibodies listed in Table S1. Negative controls include non-immunized goat, mouse, or rabbit IgG used at the same concentrations as the corresponding primary antibodies. On the following day the cells were incubated with the secondary antibody conjugated with Alexa Fluor 594 (Invitrogen, 1:400) for one hour. Cell nuclei were stained with DAPI (Sigma-Aldrich, D8417, 25 μg/ml in MilliQ water) for two minutes or with 0.2 μM SYTOX green (Invitrogen, S7020) for 30 min. Samples in Chamber Slides were mounted with a ProLong® Gold antifade reagent (Invitrogen, P36934). The protein expression was visualized on a Leica TCS SP5II HCS A confocal microscope with an HCX PL APO 20×/0.7 Imm Cor (glycerol) objective or HC PL APO 0.7 CS air objective. DAPI was excited with UV (diode 405 nm/50 mW), SYTOX green with an Argon 488 nm laser, and Alexa Fluor 594 with a DPSS (561 nm/

20 mW) laser. Emission was acquired with PMT and HyD detectors. Immunostaining of negative controls was used to setup confocal parameters, and they showed no positive staining (data not shown). The images were analyzed with Imaris 8.1.2 program (Bitplane) by creating slices or easy three-dimensional images. Immunofluorescence of the H9-GFP-derived DE cells was imaged with a Zeiss Axioplan microscope.

2.5. RNA isolation and cDNA conversion

Total RNA was extracted from HepaRG, H9-GFP cells, and their derivatives using a TRIzol reagent (Invitrogen, 15596) following the manufacturer's instructions. All the other cells were lysed with a RLT-buffer (Qiagen), and total RNA was extracted using an RNeasy Mini kit (Qiagen, 74104) according to the manufacturer's instructions. Primary human hepatocytes (PHHs; BD Biosciences, 454503, lot 99 and 95) were used as controls. Cryopreserved hepatocytes (lot 99) were from a 29 year old female donor and lot 95 from a 13 year old male donor. The frozen PHHs were recovered by using a cryopreserved hepatocyte purification kit (BD Biosciences, 454500) according to the manufacturer's instructions. The RNA was converted to cDNA with a High Capacity RNA-to-cDNA kit (Applied Biosystems, 4387406). The cDNA samples were used in conventional PCR and qPCR.

2.6. Conventional RT-PCR

The gene expression of the ECM proteins in HepaRG cells was measured by conventional PCR using a KAPA HiFi HotStart kit (KAPA Biosystems, KK2501). Each PCR reaction consisted of 0.5 U KAPA HiFi HotStart DNA polymerase, 20 ng cDNA, 0.3 μM primers, 0.3 mM dNTP mix, 1 × KAPA HiFi buffer, and PCR grade water. The primer sequences are listed in Table S2. The PCR cycles were performed on a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories). The PCR cycling conditions were: initial denaturation at 95 °C for 5 min followed by 25 cycles of 20 s denaturation at 98 °C, 15 s annealing at 60 °C, and 30 s extension at 72 °C. The PCR cycles were followed by a final extension at 72 °C for 5 min and cooling at 4 °C. The PCR products were examined by standard agarose gel electrophoresis (1% agarose, 0.5 μg/ml ethidium bromide, 1 × TBE at pH 8.0) and visualized under a UV transilluminator with a CCD camera with a motor-operated zoom lens (Syngene Gene Genius Bio Imaging System, Synoptics). The size of the PCR products was assessed by comparison with an O'GeneRuler™ Low Range DNA Ladder (Fermentas, SM1203).

2.7. qPCR

The cDNA samples were analyzed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using a Fast SYBR Green Master Mix (Applied Biosystems, 4385612) or TaqMan Universal Master Mix II (Applied Biosystems, 4440038). Housekeeping gene ribosomal protein, large, P0 (*RPLP0*) served as an endogenous control. All used primers and TaqMan Gene Expression Assay mixes are listed in Tables S2 and S3, respectively. The primers were designed by Primer Express v2.0 software (Applied Biosystems) except the primers for *OCT4* [17] and *HNF3B* [20], and were synthesized by Oligomer Oy (Helsinki, Finland). A standard curve for each gene was generated, and amplification efficiency was taken into account in calculation of the relative quantification of each target gene in comparison with the housekeeping gene with an earlier described mathematical model [22]. The relative gene expression was calculated with reference to the undifferentiated hPSCs on day 0, which represents one.

2.8. Albumin (ALB) secretion

Hepatic functionality was assessed by measuring human ALB secretion over time. Conditioned media samples of iPS(IMR90)-4 and H9-GFP cells were analyzed with a Human Albumin ELISA Quantiation Set (Bethyl Laboratories, E80-129) according to the manufacturer's protocol. To normalize secreted ALB amount, total cellular protein content was analyzed. Cells were lysed with 1 × RIPA buffer (ThermoFisher Scientific, 89901) supplemented with 1 × protease inhibitor cocktail (Sigma Aldrich, P8340) after which the protein amount was measured with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, 23227) by following the manufacturer's instruction.

2.9. Cytochrome P450 (CYP) enzyme activity and induction

The activity of CYP3A enzymes was followed over time in the hPSC-derived cells with a P450-Glo™ CYP3A Assay with Luciferin-PFBE (Promega, V84901). The cell monolayer was washed twice with basal media before adding 50 μM CYP3A substrate diluted in the basal media. After eight-hour incubation luminescence was generated by adding Luciferin Detection Reagent and the formed light was recorded with a plate reader (Varioskan Flash, Thermo Fisher Scientific) [23]. The cells were then lysed with 1 × RIPA buffer as described above to normalize the luminescence to total protein amount. The induction of *CYP3A4*, *CYP3A7*, *CYP1A1*, *CYP1A2*, and *CYP1B1* expression were examined with known inducers. The cells were exposed to 50 μM DEX (in 0.1% DMSO) for 48 h to induce *CYP3A4* and *CYP3A7* or to 100 μM omeprazole (OMZ; in 0.1% DMSO) to induce *CYP1A1*, *CYP1A2*, and *CYP1B1*. Control cells were treated with 0.1% DMSO for 48 h. RNA lysates, RNA isolation, cDNA conversion, and real-time qPCR were performed as described above.

2.10. Periodic Acid Schiff (PSA) staining for glycogen

Glycogen production was analyzed by PAS staining. The hPSC-derived cells and HepaRG cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Human liver tissue was obtained from harvested organs for liver transplantation in the Transplantation and Liver Surgery Clinic (Helsinki, Finland). Human liver tissue sample was fixed with 4% paraformaldehyde overnight at 4 °C. Subsequently, the standard paraffin embedding and sectioning were performed at the Finnish Center for Laboratory Animal Pathology. 5 μm-thick sections were used in PSA staining. The cells and deparaffinized tissue sections were treated with 0.5% periodic acid solution for 5 min. After washing with distilled water the cells were incubated with Schiff's reagent for 15 min, and the reagent was then washed away extensively with running tap water. The stained samples were imaged with a phase contrast microscope (Leica DM IL LED) with LAS EZ software (Leica Microsystems). The use of human liver tissue was authorized by the National Supervisory Authority for Welfare and Health and by the Hospital District of Helsinki and Uusimaa Ethics Committee Department of Surgery.

2.11. Statistical analysis

Statistical significance was determined by Student's t-test (Fig. 7E) or by one-way analysis of variance (ANOVA) followed by Holm-Sidak post-test (Sigma Plot 11.0; Figs. 5C, 7D, and 8). Differences of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) were considered significant.

3. Results

3.1. Characterization of the ECM proteins in HepaRG cells

We have earlier shown that the human liver progenitor HepaRG-ACM supports the attachment and hepatic differentiation of hPSC-derived DE cells [14]. To produce a chemically defined matrix that mimics the HepaRG-ACM, we characterized the ECM proteins in HepaRG cells by conventional RT-PCR and immunofluorescence. Fig. 1A summarizes the mRNA and proteins expressed by the HepaRG cells. Fibronectin, laminin α2, α5, β1, β2 and γ1 chains, and collagen type IV α1, α2 and α5 chains were detected at mRNA level (Fig. S1). Fibronectin, laminin α5 chain, and collagen type IV α2 and α5 chains were also detected at protein level as shown by immunofluorescence (Fig. 1B). Collagen type IV α2 and α5 chains cannot assemble any known heterotrimers as only three heterotrimers, namely α1α1α2, α3α4α5, and α5α5α6, have been discovered [24]. This result suggests that HepaRG cells produce fibronectin, LN-511 (composed of α5, β1, and γ1 chains), and LN-521 (composed of α5, β2, and γ1 chains) proteins.

3.2. Characterization of hPSCs and their differentiation to DE cells

Prior to DE and hepatic differentiation, the hPSC cells were characterized in terms of their pluripotency. The WA07 and iPS(IMR90)-4 cells expressed the key pluripotent markers OCT4 and SSEA-4, and H9-GFP expressed OCT4 as shown by immunofluorescence (Fig. S2A). The stem cells were negative for hepatic markers AFP, ALB, and HNF4A (data not shown). In the next step of differentiation, the cells were induced to DE cells according to earlier published protocols [14,21]. The DE cells derived from all three different hPSC lines highly expressed HNF3B and CXCR-4 (Fig. S2B) but were negative for AFP (data not shown).

3.3. Studying ECM components for hepatic differentiation

Here we tested the ECM proteins fibronectin, LN-511, and LN-521 that are found in HepaRG cells, and all their possible combinations for the attachment of DE cells to create a xeno-free, human liver progenitor-specific culture matrix. After six days of DE induction the cells were detached and transferred to the studied matrices. The DE cells were differentiated towards hepatic cells with a three-step treatment: hepatic specification, hepatic progenitor expansion, and hepatic maturation (Fig. 2A).

To identify if one of the ECM proteins found in HepaRG cells had an essential role in hepatic specification, we first plated the H9-GFP-derived DE cells onto four matrices with the combinations of two to three proteins, namely M1 (LN-511, LN-521, and fibronectin), M2 (LN-511 and LN-521), M3 (LN-511 and fibronectin), and M5 (LN-521 and fibronectin). If the differentiation potency was decreased on one of the two-protein conditions compared with the three-protein condition, the excluded protein would be an essential matrix in hepatic specification. To know the concentration of LN511 and LN-521, we tested 10 μg/ml that is recommended by the supplier and 20 μg/ml. One day after seeding the H9-GFP-derived DE cells to the four matrices, we noticed that the attached cells were much more on the matrices with 20 μg/ml LN-511 or LN-521 (Fig. S3). Therefore, in the subsequent studies, we used LN-511 and LN-521 at 20 μg/ml. On day 10, the H9-GFP-derived progenitor cells expressed HNF4A and AFP at protein level in all four studied culture conditions as observed by immunofluorescence (Fig. S4A). On day 21, the hepatic cells differentiated from the H9-GFP cells highly expressed ALB, and many cells lost the expression of AFP (Fig. S4B). We did not observe any significant differences in the expression of the hepatic markers between the matrices M1, M2, M3, and M5. The finding was confirmed with the studies of gene expression and ALB secretion. The mRNA expression of each hepatic marker (*ALB*, *AAT*, *CK-19*, *CK-8*, *CK-18*, and *AFP*) in the H9-GFP-derivatives on day 21 was at the similar level in all four

A

Matrix component	RT-PCR	IF
Fibronectin	+	+
Laminin, α1	-	NA
Laminin, α2	+	-
Laminin, α3	-	NA
Laminin, α4	-	NA
Laminin, α5	+	+
Laminin, β1	+	NA
Laminin, β2	+	NA
Laminin, γ1	+	NA
Laminin, γ2	-	NA
Laminin, γ3	-	NA
Collagen, type I, α1	-	NA
Collagen, type IV, α1	+	-
Collagen, type IV, α2	+	+
Collagen, type IV, α3	-	-
Collagen, type IV, α4	-	NA
Collagen, type IV, α5	+	+
Collagen, type IV, α6	-	NA

NA: not analyzed

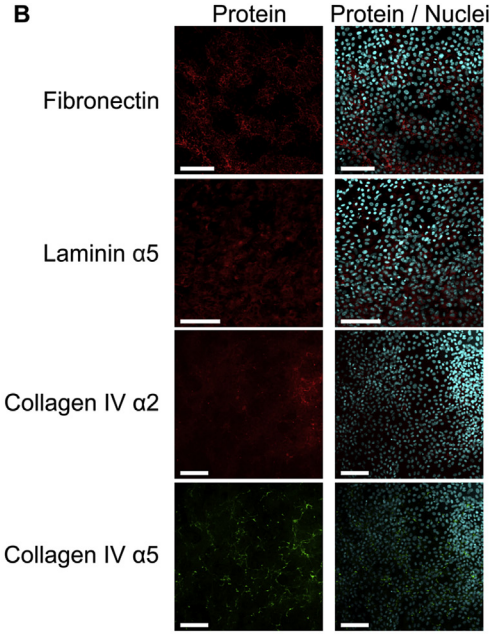
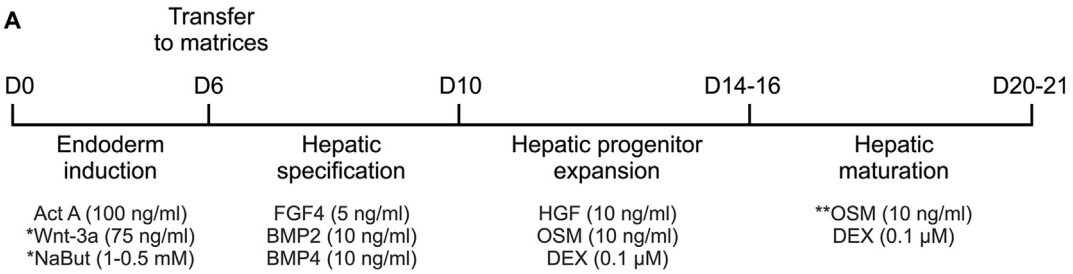


Fig. 1. Characterization of the ECM components produced by the human liver progenitor HepaRG cells. A) The summary of the ECM components analyzed in the HepaRG cells by conventional RT-PCR and immunofluorescence (IF). B) Immunofluorescence of fibronectin, laminin α5, collagen type IV α2, and collagen type IV α5 in the HepaRG cells. Scale bars = 100 μm. Controls in which the primary antibody was replaced with the corresponding non-immunized goat, mouse, or rabbit IgG at the same concentration show no positive staining (data not shown).



B

Matrix component for coating	M1	M2	M3	M4	M5	M6	M7
20 μg ml $^{-1}$ Human rLaminin-511 (BioLamina, LN511-01)	x	x	x	x			
20 μg ml $^{-1}$ Human rLaminin-521 (BioLamina, LN521-01)	x	x			x	x	
25 μg ml $^{-1}$ Fibronectin (Sigma-Aldrich, F0895)	x		x		x		x

Fig. 2. Hepatic differentiation of the hESC and hiPSC-derived DE cells in fibronectin, laminin-511, and laminin-521-based culture systems. A) Differentiation protocol. *Wnt-3a and sodium butyrate (NaBut) were used for the H9-GFP-derived DE cells. **Oncostatin M (OSM) supplement was tested with the iPS(IMR90)-4 and WA07 derivatives to improve hepatic maturation but was removed from the media as it caused cytoplasmic vacuoles extensively. B) The components used in the tested matrices M1, M2, M3, M4, M5, M6, and M7.

culture conditions (Fig. S4C).

As we were not able to identify if one of the proteins of interest had more crucial role than the others in the hepatic differentiation by screening them in combinations of two or three, we plated the hPSC-derived DE cells on matrices composing of only one protein. In addition to single protein matrices, M4 (LN-511), M6 (LN-521), and M7 (fibronectin), we repeated the cell differentiation on matrices M1, M2, M3, and M5 with iPS(IMR90)-4 and WA07 cell lines. Before an extensive study of seven matrices, we optimized the seeding density for the DE cells derived from iPS(IMR90)-4 and WA07 cells using M1. For the iPS(IMR90)-4 cells the optimal DE seeding density was 100 000 cells/cm^2 since the other tested densities, 20 000 cells/cm^2 and 50 000 cells/cm^2, did not yield as high expression level of AFP and HNF4A on day 10 (Fig. S5A). In addition, the AFP expression in the cells seeded at the density of 100 000 cells/cm^2 was the lowest on day 21 (Fig. S5C). For the WA07-derived DE cells the seeding densities 50 000 cells/cm^2 and 100 000 cells/cm^2 resulted in very similar AFP and HNF4A expression patterns on day 10 (Fig. S5B), and on day 21 no remarkable differences were observed between the studied densities (Fig. S5D). Based on these results, the experiments were continued with the density of 100 000 cells/cm^2 for the iPS(IMR90)-4 cells and with the density of 50 000 cells/cm^2 for the WA07 cells.

The hepatic specification of the iPS(IMR90)-4 and WA07-derived DE cells was efficient on most of the studied matrices. On day 10, most of the iPS(IMR90)-4 derivatives expressed HNF4A on all the matrices except on M7 as shown by immunofluorescence (Fig. 3A). The WA07-derived cells cultured on the matrices M1–M7 expressed HNF4A (Fig. 3B). On day 21, the hepatic cells derived from the iPS(IMR90)-4 and WA07 cells were partially positive for ALB (Fig. 4). The lowest ALB expression was seen in the iPS(IMR90)-4 derivatives on M2 (Fig. 4A) and in WA07 derivatives on M1 and M7 (Fig. 4B). Otherwise there was no clear difference between the studied matrices. As we did not observe any significant improvement in the hepatic specification or maturation with the presence of fibronectin, we continued the future studies with the matrices containing only laminins: M2, M4, and M6.

We plated the hPSC-derived DE cells after six days of differentiation to plastic and did not observe cell attachment (Fig. S6A). In contrast, the DE cells plated on the matrices M2, M4, and M6 attached well and formed more than 80% confluent monolayer one day after seeding (Fig. 5A and B). We did not see any difference in the DE cell attachment on day 1 when preparing the laminin coatings with the fast protocol (2 h at 37 °C) or with slow coating (overnight at 4 °C; Fig. S6B). In addition, we observed that the laminin working solutions can be reused at least two times without causing any difference in the cell attachment (Fig. S6C).

3.4. Characterization of hPSC-derived hepatic cells

After screening the optimal DE seeding density and ECM proteins we continued the study with iPS(IMR90)-4 cell line and partially with WA07 cell line using the matrices M2, M4, and M6. We followed the differentiation by examining cell morphology, hepatic marker expression at mRNA and protein levels, and hepatic functions.

The cells exhibited typical morphologies during SC, DE, and hepatic differentiation steps (Fig. 5A and B). The cell size greatly increased during the differentiation from day 10 to day 16 (Fig. 5A and B), which is a phenomenon occurring during the maturation of hepatocytes *in vivo* [25]. Already on day 16, the derived cells exhibited polygonal-shaped morphology, and some cells were binucleated. The morphology of the derived hepatic cells resembled earlier described hESC-derived hepatocytes [10,26] and PHHs in culture [27–29].

To understand the cell-matrix interactions, we studied the expression of integrin subunits *ITGA3*, *ITGA6*, *ITGA7* (including two splice variants *ITGA7X1* and *ITGA7X2*), *ITGB1*, and *ITGB4* in the iPS(IMR90)-4 cells, DE cells (day 6), and derived cells on day 10 corresponding to four-day culture on the studied matrices M2, M4, and M6. These integrin subunits can form the major laminin-binding integrins α3β1, α6β1, α7X1β1, α7X2β1, and α6β4. In general, the integrin expression profiles changed drastically between

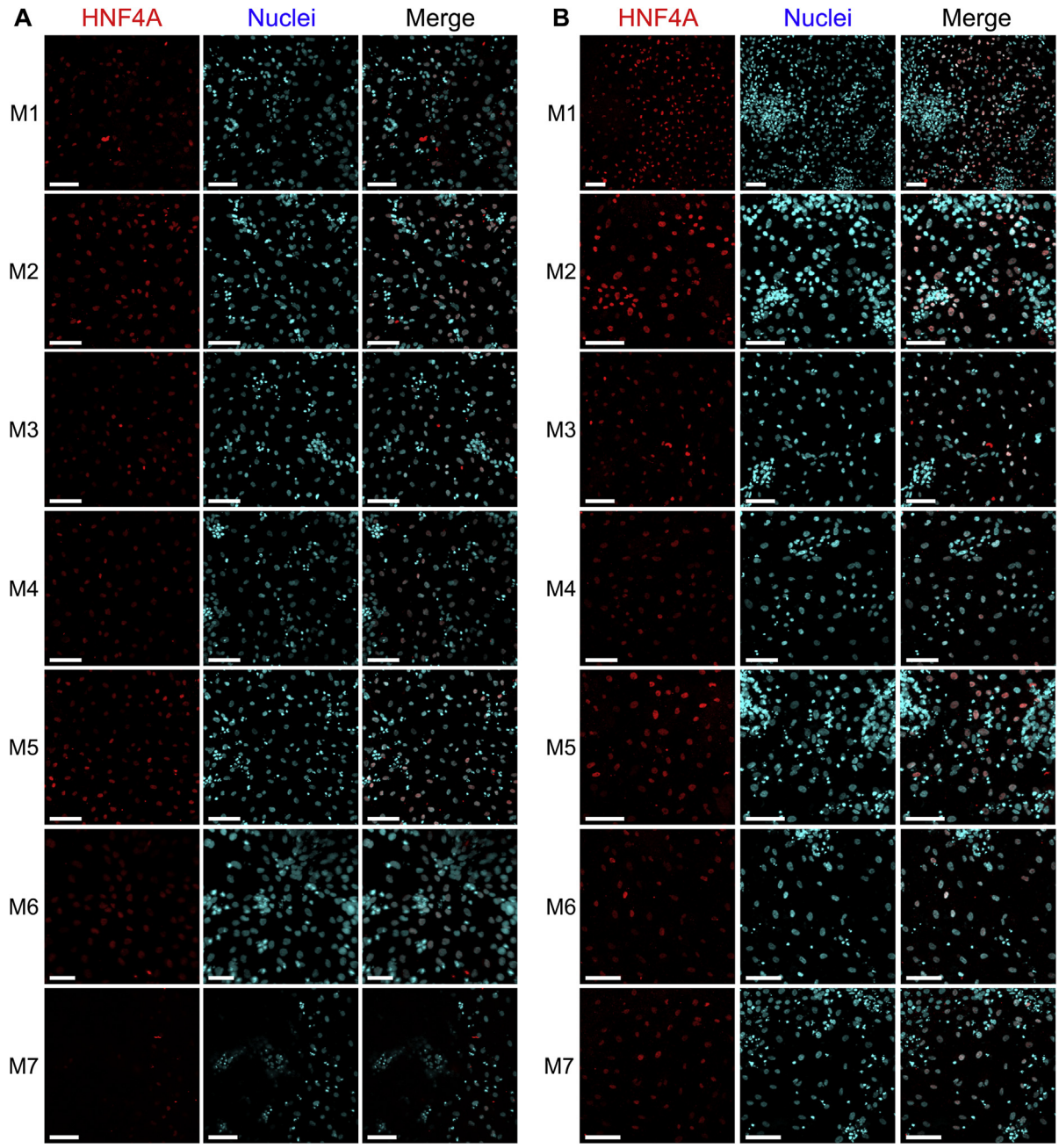


Fig. 3. Hepatic specification of the hPSC-derived DE cells on the seven matrices. Immunofluorescence of HNF4A in A) the iPS(IMR90)-4-derived progenitor cells and B) WA07-derived progenitor cells on day 10. Scale bars = 100 μm. Controls in which the primary antibody was replaced with the corresponding non-immunized goat IgG at the same concentration show no positive staining (data not shown).

the analyzed time points (Fig. 5C). The expression of integrin α subunit *ITGA3* significantly increased on day 10 compared with the hPSCs and DE cells. In turn, its expression in the PHHs was lower than that in the hPSCs. A similar expression pattern was observed with α subunit *ITGA7X1* and β subunit *ITGB4* whose expression was the highest in the derived cells on day 10. Compared with the hPSCs the expression of *ITGA6* and *ITGA7X2* mRNA in the DE cells and derived cells on day 10 was significantly lower, which was similar to the level in the PHHs. The expression of *ITGB1* significantly increased in the derived cells on day 10, which was similar to the level in the PHHs. The expression of the studied integrins were at the same levels between the cells cultured on M2, M4, and M6 except for *ITGB1* whose expression was significantly higher on M6 compared with M4 ($P < 0.05$).

The iPS(IMR90)-4-derived progenitors on M2, M4, and M6 expressed CK-19 and AFP on day 10 (Fig. 6A). The AFP expression on M6 was weak. At the last studied time point, on day 21, the iPS(IMR90)-4 derivatives were partially positive for AFP. Although CK-19 was still expressed, it did not exhibit clear filaments as seen on day 10 (Fig. 6B). The hepatocyte-specific marker CK-18 was expressed in all the studied matrices on day 21. The expression of the hepatic markers HNF4A and ALB in the iPS(IMR90)-4-derived cells is described in section 3.3 (Figs. 3 and 4).

Both the iPS(IMR90)-4-derived and H9-GFP-derived cells secreted human ALB confirming their hepatic functions (Fig. 7A). In the H9-GFP-derived cells the ALB secretion drastically increased

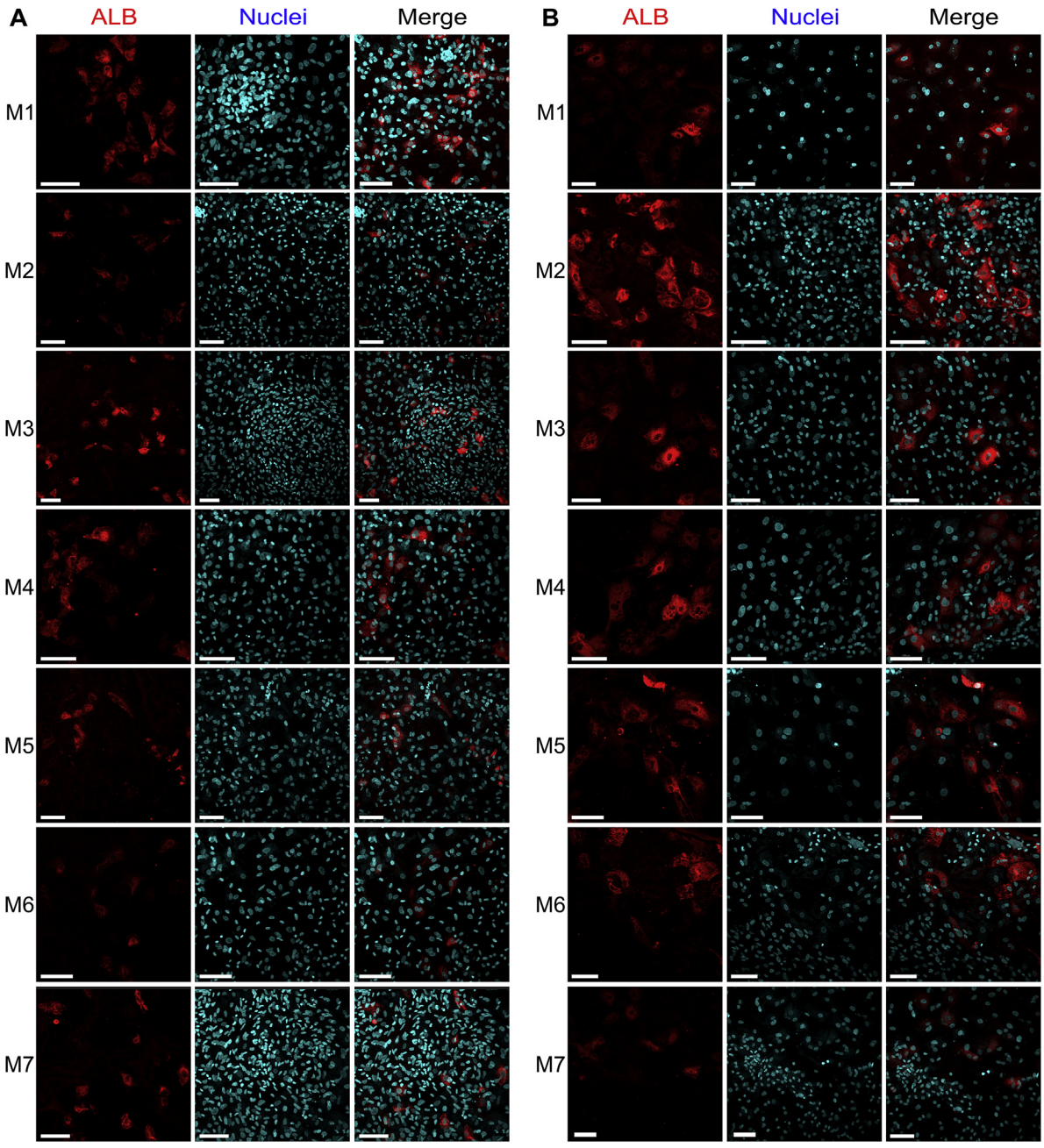


Fig. 4. Hepatic differentiation of the hPSC-derived DE cells on the seven matrices. Immunofluorescence of ALB in hepatocyte-like cells derived from A) the iPS(IMR90)-4 cells and B) WA07 cells on day 21. Scale bars = 100 μm. Controls in which the primary antibody was replaced with the corresponding non-immunized goat IgG at the same concentration show no positive staining (data not shown).

from day 16 to day 21 indicating the hepatic maturation. In the iPS(IMR90)-4-derived cells the ALB secretion could not be detected on day 10 yet, but it increased at the next studied time points, days 16 and 20.

Glucose metabolism was characterized by staining the stored glycogen with PAS. Similarly to hepatocytes in the human liver, the iPS(IMR90)-4-derived cells showed positive PAS staining (Fig. 7B). The iPS(IMR90)-4-derived hepatic cells cultured on M6 were more positive for PAS on day 21 compared with HepaRG cells.

The hepatocyte phenotype of the derived cells was also studied in terms of their detoxification functions. CYP3A4 protein was strongly expressed in the iPS(IMR90)-4-derived cells on day 21 cultured on M4 and M6, but its expression was remarkably lower on M2 (Fig. 7C). CYP3A enzyme activity significantly increased during the differentiation period, and on the last studied time point the activity was significantly higher than that in HepaRG cells (Fig. 7D). Inducibility of CYP enzymes were studied with known inducers, omeprazole (OMZ; for CYP1A1, CYP1A2, and CYP1B1) and dexamethasone (DEX; for CYP3A4 and CYP3A7). The treatment with OMZ induced the mRNA expression of *CYP1A1* and *CYP1B1* in the iPS(IMR90)-4-derived cells cultured on M6 by approximately 1000 times and 12 times compared with DMSO-treated control cells, respectively (Fig. 7E). The *CYP1A1* induction fold change was only one third in HepaRG cells compared with the iPS(IMR90)-4-derived hepatic cells (Fig. 7E). The expression of *CYP1B1* mRNA was induced at the similar levels both in the iPS(IMR90)-4-derived

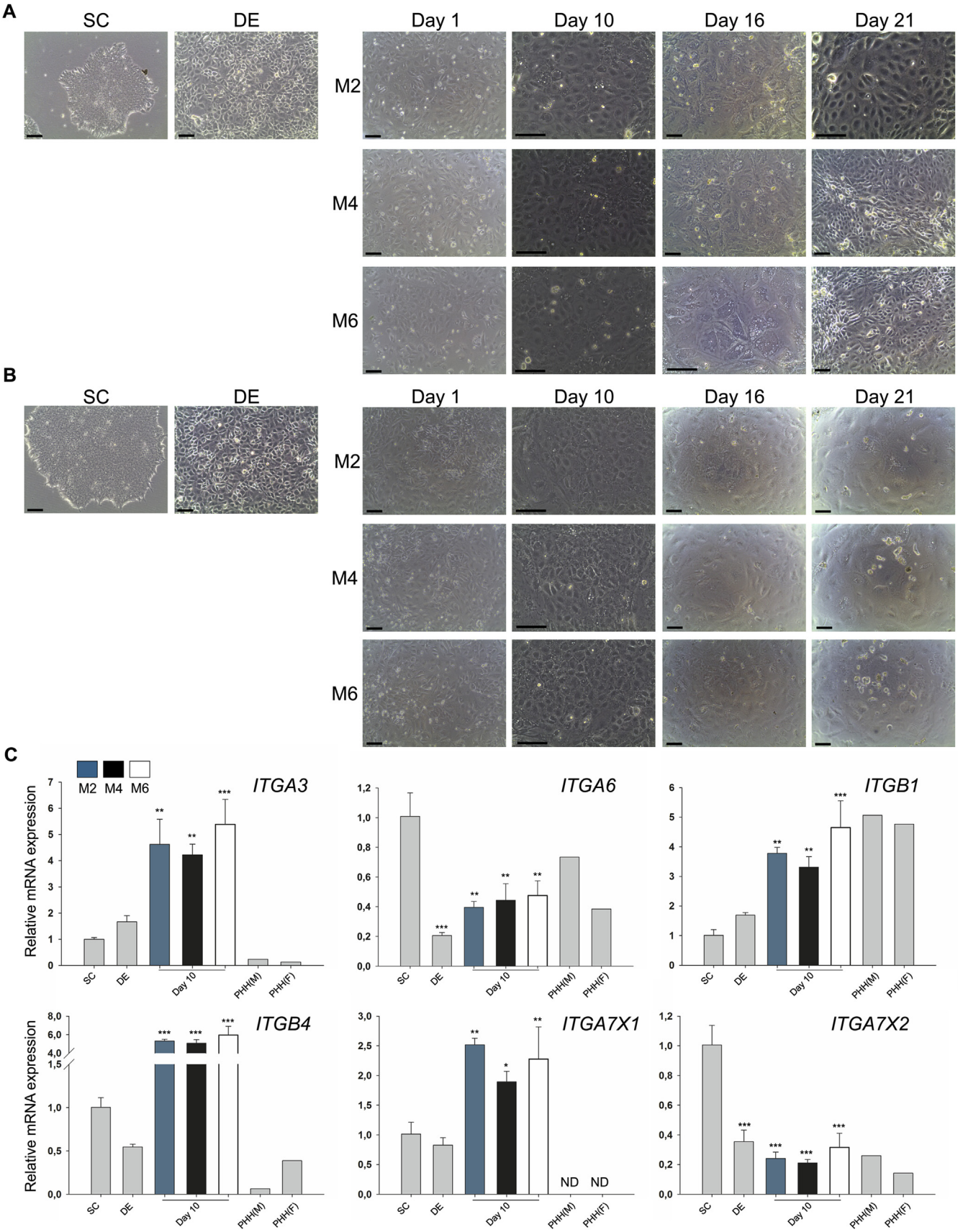


Fig. 5. Phase contrast microscope images of the hPSCs, DE cells, and their hepatic derivatives on M2, M4, and M6 at different time points. A) The iPS(IMR90)-4 cells. B) The WA07 cells. Scale bars = 100 μm. C) Expression profile of integrin subunits analyzed by real-time qPCR in the iPS(IMR90)-4 cells (SC), DE cells on day 6, and derived cells on day 10 corresponding to four-day culture on the studied matrices M2, M4, and M6. The relative mRNA expression of integrin subunits *ITGA3*, *ITGA6*, *ITGB1*, *ITGB4*, *ITGA7X1*, and *ITGA7X2* was normalized to the control gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated stem cells on day 0 (SC). N = 3 biological samples. Error bars represent SD. The differences between the iPS(IMR90)-4 cells and their derivatives were analyzed by one-way ANOVA followed by Holm-Sidak post-test. The statistically significant differences are shown as $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. PHH(M): primary human hepatocytes (BD Biosciences, 454503, lot 95); PHH(F): primary human hepatocytes (BD Biosciences, 454503, lot 99). ND = not detectable.

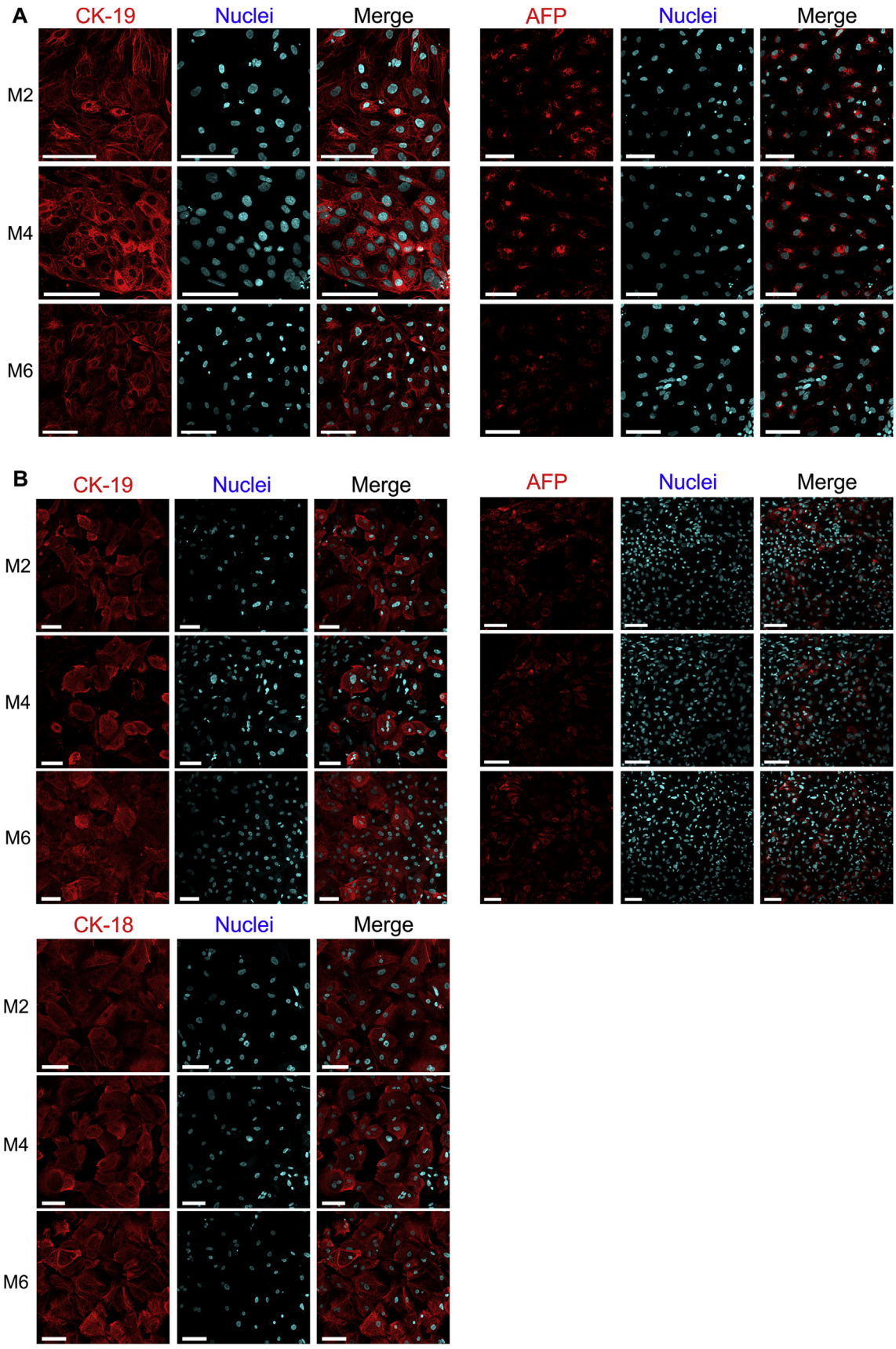


Fig. 6. Immunofluorescence staining of CK-19, AFP, and CK-18 in the iPS(IMR90)-4-derived cells cultured on M2, M4, and M6. A) The hepatic progenitor cells on day 10. B) The hepatocyte-like cells on day 21. Scale bars = 100 μm. Controls in which the primary antibody was replaced with the corresponding non-immunized mouse IgG at the same concentration show no positive staining (data not shown).

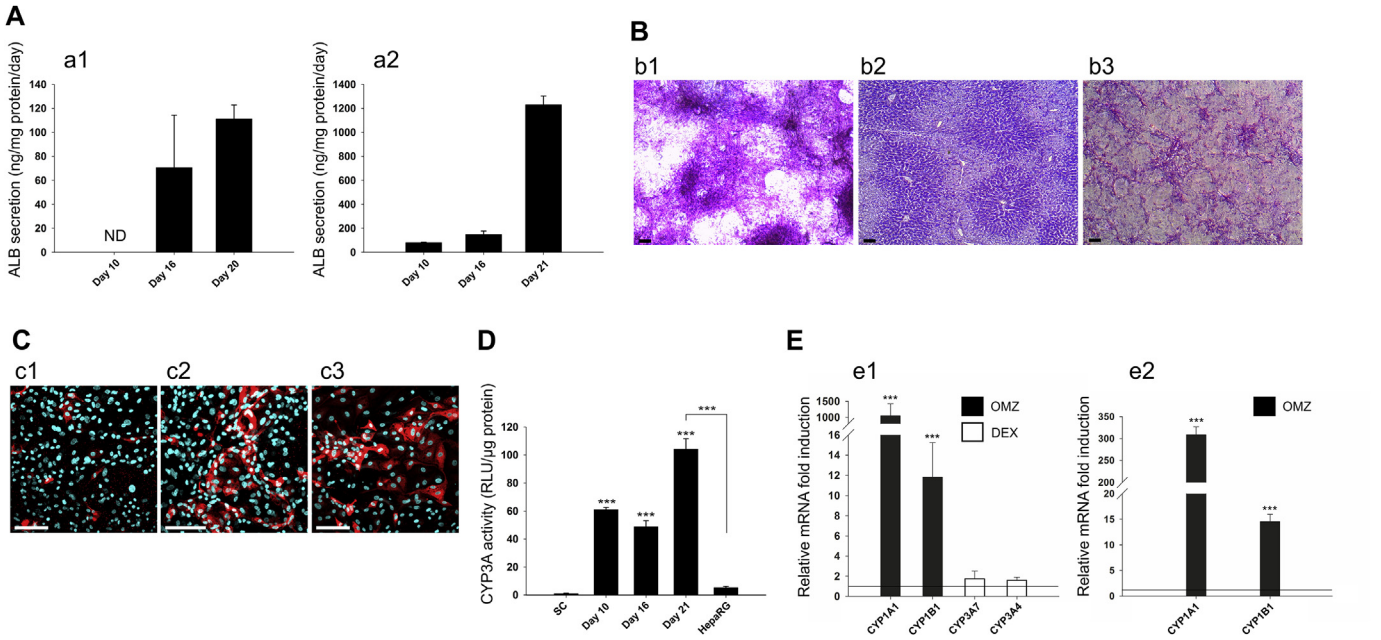


Fig. 7. Hepatic synthetic functions, energy metabolism, and detoxification function in the hPSCs-derived cells. A) ALB secretion in the iPS(IMR90)-4-derived cells (a1) and in the H9-GFP-derived cells (a2) cultured on M2. For iPS(IMR90)-4 derivatives N = 3 biological samples and for H9-GFP derivatives N = 2 biological samples. Error bars represent SD (a1) and the minimal and maximal values (a2). B) Glycogen production in the iPS(IMR90)-4-derived hepatic cells on day 21 cultured on M6 (b1), in a human liver (b2), and in the HepaRG cells (b3) as shown by PAS staining. Scale bars = 100 µm. C) Immunofluorescence of CYP3A4 enzyme in the iPS(IMR90)-4-derived cells on day 21 cultured on M2 (c1), M4 (c2), and M6 (c3). Scale bars = 100 µm. Controls in which the primary antibody was replaced with the corresponding non-immunized rabbit IgG at the same concentration show no positive staining (data not shown). D) Metabolic activity of CYP3A enzymes in the iPS(IMR90)-4 cells and their derivatives during the hepatic differentiation. The differences between all pairs were analyzed by one-way ANOVA followed by Holm-Sidak post-test. The statistically significant differences between the iPS(IMR90)-4 cells (SC) and their derivatives are shown above bars as $^{***}P < 0.001$. The statistically significant difference between the cells on day 21 and HepaRG cells is shown above lines as $^{***}P < 0.001$. For stem cells N = 3 biological samples, and for the iPS(IMR90)-4-derivatives and HepaRG cells N = 4 biological samples. Error bars are SD. E) Induction of CYP enzymes with omeprazole (OMZ) and dexamethasone (DEX) in the iPS(IMR90)-4-derived cells on day 21 cultured on M6 (e1) and in HepaRG cells (e2). N = 4 biological samples. Error bars are SD. The line represents DMSO-treated control cells = 1. The difference between the DMSO-treated control cells and inducer-treated cells was analyzed by Student's *t*-test. The statistically significant differences are shown as $^{***}P < 0.001$.

cells and in HepaRG cells after OMZ treatment. *CYP1A2* was expressed in the derived cells after the induction but not detectable in the control cells (data not shown). Exposure to DEX upregulated the mRNA expression of *CYP3A7* and *CYP3A4* in the cells cultured on M6 by 1.7 and 1.6 times compared with the control cells, respectively (Fig. 7E). However, the induction fold was not statistically significant.

3.5. Gene expression profiles of hPSCs and their hepatic derivatives

We performed comprehensive gene expression profiling with iPS(IMR90)-4 cell line by assessing the markers for pluripotent cells, DE cells, hepatic progenitors, fetal hepatocytes, and mature hepatocytes by qPCR (Fig. 8). The mRNA expression of the iPS(IMR90)-4 derivatives cultured on M2, M4, and M6 was compared with stem cells to show the relative expression. The PHHs and HepaRG cells were used as controls. The mRNA expression of the key pluripotent marker *OCT4* in the iPS(IMR90)-4 derivatives after 14 days in culture in all the studied culture matrices was at the similar level as in the PHHs. The *NANOG* expression significantly decreased from the stem cell stage already on day 10.

The mRNA expression of *HNF3B* (also known as *FOXA2*) was 240-fold upregulated in the DE cells on day 6 after which the gene expression was significantly downregulated by 100 times already in the hepatic progenitors on day 10. At the last studied time point, on day 21, the *HNF3B* expression was nearly at the same level as in stem cells, and remarkably lower than the levels in the PHHs and HepaRG cells. The gene expression of the liver progenitor marker *AFP* increased from the stem cell level approximately 80–120 times on day 10, and reached the maximal expression on day 14. On day 21, the *AFP* expression was downregulated but the level of its expression in all the studied matrices was higher than that in the PHHs. *CK-19* is a marker for hepatic progenitors and cholangiocytes [11], and it is also present in fetal hepatocytes [30]. In the iPS(IMR90)-4-derived hepatic progenitors the *CK-19* expression increased circa 25 times from the stem cell stage and remained nearly constant on day 14. On day 18, the expression was downregulated approximately 10 folds to the level which was maintained until the end of the culture. The expression of the liver specific cytokeratins *CK-8* and *CK-18* was the highest on day 14 in all three culture conditions, and later downregulated on day 18. On the last studied time point, the expression levels of *CK-8* and *CK-18* were approximately 6 times and 5 times higher than those in the control PHHs, respectively.

The expression of *CYP3A7*, expressed in fetal hepatocytes [31] but only weakly in adult hepatocytes *in vivo* [11], was upregulated in the iPS(IRM90)-4-derived cells on all the matrices M2, M4, and M6. On day 21, the *CYP3A7* expression was at the similar level to the PHHs from a 13 year old male donor, lower than in the PHHs from a 29 year old female donor, and higher than in HepaRG cells. We observed also the upregulation of *CYP3A4* expression, an enzyme present only in mature hepatocytes [11], but the level of its expression was significantly lower compared to those in the PHHs and HepaRG cells. The mRNA expression of *CYP2C9* reached the highest level on day 14 after which the expression level was stable. *CYP2B6* was not expressed in the iPS(IMR90)-4-derived cells at any stage (data not shown). We determined the expression of nuclear receptors *NR3C1* (also known as glucocorticoid receptor, *GR*), *NR1I2* (also known as pregnane X receptor, *PXR*), and aryl hydrocarbon receptor (*AhR*). NR3C1 and NR1I2 mediate the induction of CYP3A

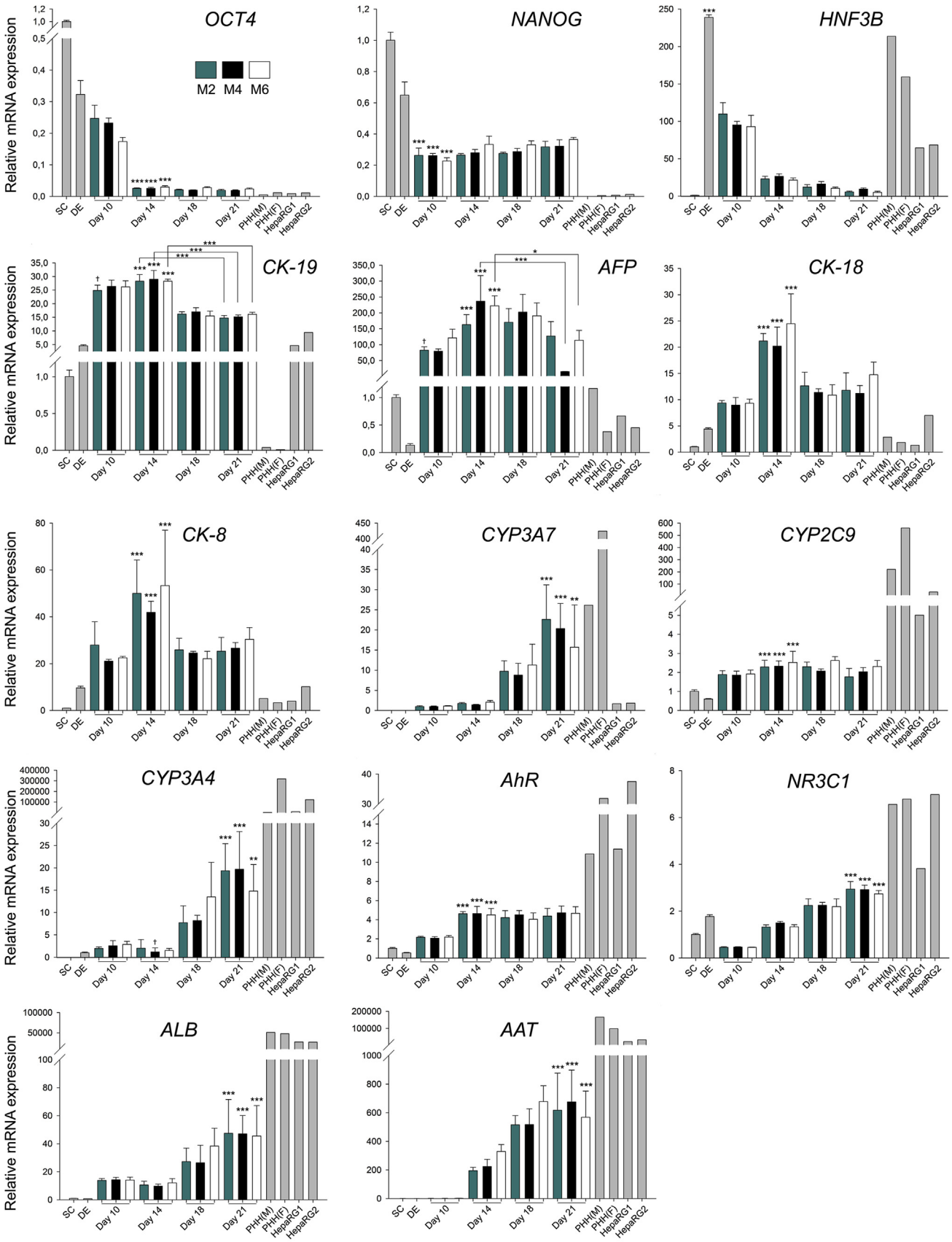


Fig. 8. Relative mRNA expression of pluripotency (*OCT4* and *NANOG*), DE (*HNF3B*), and liver (*CK-19*, *AFP*, *CK-18*, *CK-8*, *CYP3A7*, *CYP2C9*, *CYP3A4*, *AhR*, *NR3C1*, *ALB*, and *AAT*) markers in the iPS(IMR90)-4 cells during the differentiation analyzed by real-time qPCR. Relative mRNA expression was normalized to the control gene *RPLP0*, and fold inductions were calculated with reference to the undifferentiated stem cells on day 0. For stem cells and DE cells, N = 3 biological samples. For hepatic derivatives on days 10, 14, 18, and 21, N = 4

[32–36]. AhR mediates the induction of CYP1A and CYP1B1 [37]. The mRNA expression of *NR3C1* was upregulated during the differentiation, and on day 21 the level was approximately two times lower than the levels in the PHHs or DMSO-treated HepaRG cells. The *AhR* expression increased over four folds during the culture. The *NR1I2* was not expressed in the iPS(IMR90)-4 cells (data not shown).

The hepatic differentiation of the iPS(IMR90)-4 cells was further confirmed by the increased *ALB* and alpha-1 antitrypsin (*AAT*) expression during the culture. On the last day of culture, both the *ALB* and *AAT* mRNA levels were significantly higher than in stem cells, but lower than the expression in the PHHs and HepaRG cells.

4. Discussion

Cells start to secrete ECM proteins already at the embryonic stage [2]. The ECM has an essential role in the regulation of cell differentiation and cell functions and in the maintenance of tissue homeostasis [38]. ECM remodeling is an important mechanism by which tissues and organs are formed [1,3]. We hypothesized that hepatic specification of hPSC-derived DE cells would be efficient in hepatic progenitor-like environment. We earlier showed that HepaRG-derived ACM was a promising matrix for hepatic differentiation of hPSC-derived DE cells [14]. To create a xeno-free and chemically defined differentiation platform for DE cells, in this study, we first characterized the ECM components of HepaRG cells and then used the discovered components in hepatic specification of hPSC-derived DE cells.

4.1. HepaRG cells produce fibronectin, LN-511, and LN-521

In the liver, the ECM chemistry varies during the development and exhibits gradient from periportal (zone 1) to pericentral (zone 3) zones [25]. In zone 1 the matrix is composed of laminins, collagen types III and IV, hyaluronans, heparin sulfate proteoglycans, and chondroitin sulfate proteoglycans [25,39,40]. In zone 3 the matrix is dominated by collagen type I and fibronectin. At least eight intrahepatic maturational lineage stages have been suggested along the three zones [40], and they carry out distinct functions [41]. Human hepatic stem cells [42] are precursors to hepatoblasts that give rise to hepatocytes and cholangiocytes. Both hepatic stem cells and hepatoblasts are located in periportal zone 1 in the liver [43]. The liver ECM is dynamic. *In vivo* studies have revealed a transient increase in laminins in regenerating liver and that cells from regenerating liver attached better on laminins than cells from normal liver [44].

We detected fibronectin in the HepaRG cells by both conventional RT-PCR and immunostaining. Fibronectin is a multidomain protein that has a capacity to bind to cell surface receptors, collagens, and other fibronectin-binding molecules [45]. As fibronectin is found in most of human tissues [46], it has been widely used as a coating material for culturing different cell types. Fibronectin has earlier shown to promote self-renewal of hPSCs [47]. Mouse ESCs cultured on fibronectin coating were successfully differentiated to DE-like cells [48]. When fibronectin was used for plating hepatic progenitors, it did not support their attachment and caused rapid cell death [25]. Fibronectin supported the attachment of normal mouse liver cells [44]. These findings indicate that fibronectin, a zone 3 matrix protein, can be used to culture mature hepatocytes but it may not be ideal for hepatic progenitors. In our study, we observed that DE cells attached to fibronectin and the cells could be further differentiated to hepatocyte-like cells. However, as fibronectin is not a liver specific ECM protein and we did not observe any improvement in the cell differentiation efficacy, we did not include fibronectin in our later studies.

We also found that HepaRG cells expressed collagen type IV α1, α2, and α5 chains at mRNA level, but the expression of α1 chain could not be confirmed at protein level. Collagen type IV, composed of three α-chains (α1–α6), is found only in the basement membranes [24]. Collagen type IV found in the vascular basement membranes and in the space of Disse in the liver is mostly composed of α1 and α2 chains while the expression of the α3 and α5 chains has been reported to be low [49]. So far, only collagen type IV proteins α1α1α2, α3α4α5, and α5α5α6 have been discovered in mammalian cells [24]. Since the identified collagen type IV α2 and α5 chains cannot assemble any known collagen type IV protein, we did not use collagen type IV in our study.

Laminins, originally found in a mouse tumor in 1979 [50], are ECM proteins present predominantly in the basement membrane in most tissues in human [51,52]. These multidomain heterotrimers are named on the basis of their α, β, and γ chains [53]. Up to date, 18 laminin isoforms have been described [51]. The laminin isoforms have tissue specific locations, and they are vital for a number of physiological functions. In this study, we found that HepaRG cells produced both LN-511 and LN-521 confirmed by immunostaining. Our finding of the presence of laminin α5, β1, and β2 chains is in agreement with an earlier finding in human liver [4]. Recombinant human LN-511 (composed of α5, β1, and γ1 chains; formerly known as laminin-10) has earlier been used for culturing mouse PSCs [54] and hPSCs [55], and human corneal endothelial progenitor cells [56]. LN-521 (composed of α5, β2, and γ1 chains; formerly laminin-11) has been applied as a culture matrix for maintaining hPSCs [18] and somatic cells [57], and for hiPSC reprogramming [58] and hiPSC differentiation towards cell types such us cardiomyocytes [59] and dopaminergic neurons [58].

Taken together, we have discovered that HepaRG cells secrete abundantly zone 1 matrix proteins but only fibronectin from the class of zone 3 matrix proteins. It has previously been described that the ECM components from zone 1 to zone 3 have dissimilar effects on human liver progenitors *in vitro* [25].

4.2. LN-511 and LN-521 support efficient hepatic specification of DE cells

Next, we used the identified matrix components in the hepatic specification of hPSC-derived DE cells. We found that LN-511 and LN-521 supported efficient hepatic specification of hPSC-derived DE cells. The H9-GFP, iPS(IMR90)-4, and WA07 cell lines differentiated into HNF4A-expressing hepatic progenitor cells after four days on LN-511 and LN-521. The other liver progenitor markers CK-19 and AFP were also highly expressed in the derived cells on day 10. The iPS(IMR90)-4 and WA07-derived hepatocyte-like cells were partially positive for ALB and CYP3A4 as observed by immunofluorescence. The cells clearly changed their lineage state towards maturation as the expression of *AFP* and *CK-19* was significantly decreased from day 14 to day 21 shown by qPCR. All the derived hepatic cells showed increased ALB secretion. We also characterized the energy metabolism functions in the derived cells by

biological samples. † N = 3 biological samples. Error bars represent SD. The differences between all pairs were analyzed by one-way ANOVA followed by Holm-Sidak post-test. The statistically significant differences between the iPS(IMR90)-4 cells (SC) and their derivatives are shown above bars as $^{**}P < 0.01$ or $^{***}P < 0.001$. The statistically significant differences between the derivatives at two time points are shown above lines as $^{*}P < 0.05$ or $^{***}P < 0.001$. PHH(M): primary human hepatocytes (BD Biosciences, 454503, lot 95); PHH(F): primary human hepatocytes (BD Biosciences, 454503, lot 99); HepaRG 1: standard two-week culture; HepaRG 2: four-week culture with 2% DMSO during the last two weeks.

staining stored glycogen. Positive PAS staining in the iPS(IMR90)-4-derived hepatic cells indicates glycogen synthesis, which is one of the key functions of hepatocytes.

Comparing with our earlier study using the HepaRG-ACM [14], we have found that the use of recombinant matrix proteins is faster, more consistent, more efficient, and more scalable. In the ACM-based system, the derived cells on day 10 were less positive for HNF4A and negative for AFP, and the cells on the final culture day were much less positive for ALB and CYP3A4 [14]. The lower differentiation efficiency in the ACM-based system could be due to the loss of the matrix proteins during decellularization and variations in matrix productions during two-week culture of the HepaRG cells. The matrix concentrations in the ACM cannot be well determined, whereas the use of recombinant matrix proteins can be easily standardized. We believe that the system presented in this study is more advanced than our previously reported ACM-based system.

Mouse and human ES cells have earlier been cultured on an ACM prepared from human embryonic kidney cell line stably expressing LN-511 followed by differentiation into hepatic lineages [60] and pancreatic lineages [61]. A recent study showed hepatic differentiation of hESCs on LN-521 [62]. Mouse hepatocytes cultured on a LN-511/521-containing ACM maintained their liver-specific functions longer than the cells cultured on Matrigel [63]. Best to our knowledge, LN-511 and LN-521 have not been used earlier for the differentiation of hPSC-derived DE cells.

Integrins are the main type of cell adhesion receptors that animal cells use to bind to the ECM [64]. They are heterodimeric molecules composing of an α and a β subunit. We studied the expression of the integrin subunits *ITGA3*, *ITGA6*, *ITGA7* (including two splice variants *ITGA7X1* and *ITGA7X2*), *ITGB1*, and *ITGB4*, which can form integrins α3β1, α6β1, α7X1β1, α7X2β1, and α6β4. All of these integrins are specific receptors for laminins except for α3β1 that also binds to thrombospondin [65,66]. We have noticed that the expression of the major laminin-binding integrins during the hepatic differentiation is dynamic. The hPSCs express wide variety of LN-specific integrins, which can explain why hPSCs can be cultured on either Matrigel, LN-511, or LN-521. On the other hand, compared with the SCs, the DE cells and derived cells on day 10 have less variety of laminin-specific integrins, limited to α3β1 and α7X1β1. Nishiuchi et al. reported that α3β1, α6β4, and α7X1β1 integrins have clear binding specificity to LN-511 and LN-521 [66]. Thus, α3β1 and α7X1β1 presumably mediated the binding of the DE and derived cells on day 10 to LN-511 and LN-521 in this study. Upon DE induction, the cells expressed lower levels of *ITGA6* and *ITGA7X2* than the hPSCs. Integrins α6β1 and α7X2β1 mediate the binding to LN-111 [66,67]. This indicates that the DE cells have lower capability to bind to LN-111 than the hPSCs, which can explain our earlier observation that the hPSC-derived DE cells poorly attached to LN-111 and to Matrigel which is mainly composing of LN-111 [14]. We also noticed that the PHHs expressed restricted combinations of LN-specific integrins, mainly α6β1 and weakly α7X2β1. This expression profile is distinct from that in the SCs, DE cells, and derived cells on day 10. This is in agreement with an earlier finding that integrin expression pattern markedly alters during the embryonic liver development [68]. Altogether, the dynamic expression of integrins during hepatic differentiation and liver development supports our claim that stage-specific matrices in hepatic differentiation are required.

Biotransformation of endogenous and exogenous compounds is one of the central functions of hepatocytes [69]. CYPs are vital mono-oxygenase enzymes in transforming drugs and other chemicals into more hydrophilic form and, thus, facilitating excretability. Prediction of possible drug-induced hepatotoxicity is one of the critical tasks at the preclinical stage of drug development. Human primary hepatocytes serve as the gold standard for *in vitro* tests [70,71] of drug biotransformation and liver toxicity even though their limitations in terms of availability, rapid loss of metabolizing capacity, and batch-to-batch variations are well recognized. Together with hepatic cell lines, hPSCs offer a promising source of hepatocytes for the *in vitro* models. Indeed, only viable cells, not subcellular fractions such as microsomes, can be used to study the whole biotransformation pathways of drugs and chemicals. We assessed the mRNA expression of several CYPs and their regulatory nuclear receptors by qPCR and the protein expression of CYP3A4 by immunofluorescence staining, and also performed functional studies. During the differentiation of the iPS(IMR90)-4 cells, we observed the upregulation of *NR3C1* and *AhR* expression, a nuclear receptor responsible for DEX-mediated induction of CYP3A isoforms and OMZ-mediated induction of CYP1A1, CYP1A2, and CYP1B1 enzymes, respectively. Thus, we tested inducibility of *CYP3A4* and *CYP3A7* by DEX treatment and inducibility of *CYP1A1*, *CYP1A2*, and *CYP1B1* by OMZ treatment. OMZ significantly induced the expression of *CYP1A1* and *CYP1B1* mRNA expression in the derived hepatic cells. Also *CYP1A2* mRNA expression was induced during OMZ treatment but the induction fold could not be calculated as the enzyme was not expressed without the OMZ exposure. We earlier noticed that *CYP1A2* was not expressed in the commercial hPSC-derived hepatocyte-like cells (unpublished observation). Also DEX induced the expression of *CYP3A7* and *CYP3A4* in the hiPSC-derived cells cultured on M6. The expression of *CYP3A4* and *CYP3A7* mRNA in the iPS(IMR90)-4 derived hepatic cells was significantly increased during the differentiation, and the expression of *CYP3A7* mRNA reached similar levels as in the PHHs. The activity of CYP3A enzyme was also significantly increased during the hepatic differentiation of the iPS(IMR90)-4 cells, higher than that in HepaRG cells.

Taken together, the derived cells have gained hepatic functions including synthetic functions, energy metabolism functions, and detoxification functions even though they still exhibited some fetal hepatocyte phenotypes. Future work to improve maturation could focus on the matrix composition and topology required by mature hepatocytes. Based on our current integrin data, the ideal matrices for hepatic maturation could be different from that for hepatic specification of DE cells, presumably the liver zone 3 matrix components. Intensive study of integrins and other matrix receptors is required for choosing right matrices. *In vivo*-like microenvironmental cues provided by biomaterials and adjacent cells in a defined configuration could have beneficial effects on hepatic maturation as suggested recently [11,13].

5. Conclusions

The ECM plays an important role in guiding the intrahepatic lineage specification and maturation during liver development and regeneration. Thus, mimicking the stage-specific environment *in vitro* is essential for bridging the gap between cell cultures and *in vivo*. In this study we have discovered that liver zone 1 matrix proteins, LN-511 and LN-521, promote the hepatic specification of both hESC and hiPSC-derived DE cells. The derived cells cultured on LN-511 or LN-521 are competent to acquire certain hepatic functions. The dynamic expression of integrins during hepatic differentiation supports our claim that stage-specific matrices are required to better control differentiation.

Conflict of interest

All authors confirm that there are no conflicting interests.

Author contribution

Y.-R.L. conceived and designed the research; Y.-R.L. and L.K.K. designed the experiments; L.K.K., R.H., P.P., M.S.B., T.S., P.P., J.N., T.S., and Y.-R.L. carried out the experiments; Y.-R.L. and L.K.K analyzed the data; M.Y. and A.U. commented on the research; L.K.K. and Y.-R.L. wrote the paper; all the authors commented on the final version of the paper.

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Appendix A. Supplementary data

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