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

Liisa K. Kanninen [a], Riina Harjumäki [a, 1], Pasi Peltoniemi [a, 1], Mariia S. Bogacheva [a], Tuuli Salmi [a], Pauliina Porola [a], Johanna Niklander [a], Tomáš Smutný [a, b], Arto Urtti [a, c], Marjo L. Yliperttula [a], Yan-Ru Lou [a, *]

[a] Division of Pharmaceutical Biosciences, Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, P. O. Box 56, FI-00014 Helsinki, Finland
[b] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Charles University in Prague, 50005 Hradec Králové, Czech Republic
[c] School of Pharmacy, University of Eastern Finland, P. O. Box 1627, 70211 Kuopio, Finland

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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.

* Corresponding author.
E-mail address: yan-ru.lou@helsinki.fi (Y.-R. Lou).
[1] These two authors contributed equally to this work.

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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/