BIOINSPIRED MATRICES FOR IN VITRO HEPATIC DIFFERENTIATION

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Bioinspired matrices for in vitro hepatic differentiation

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ACADEMIC DISSERTATION

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Abstract

Standard two-dimensional (2D) in vitro cell culture systems do not mimic the complexity found in the liver as three-dimensional (3D) cell-cell and cell-matrix interactions are missing. Although the concept of cell culturing was established over 100 years ago the currently used culture techniques are not yet ideal. In the field of pharmacy especially, the need of physiologically-relevant models to characterize biotransformation pathways during drug development is urgent. Hepatocytes, the main cell type of the liver, are essential components in these in vitro models. Liver cell lines and derivation of hepatocyte-like cells from stem cells are alternative sources to primary isolations for obtaining hepatocytes.

In the liver, hepatocytes are in continuous interaction with other cells and surrounding extracellular matrix (ECM). Moreover, liver functions are strictly dependent on correct tissue architecture. One approach to improve the standard cell culture systems is to mimic the hepatocytes' natural microenvironment and organization by culturing the cells within biomaterial matrices. Matrix-based culture systems for hepatocytes have been developed from natural, synthetic and hybrid biomaterials and the cells can be grown in 2D or 3D configuration. The aim of this thesis was to find new defined culture matrices for in vitro hepatic differentiation.

First, we studied two biomaterials, nanofibrillar cellulose (NFC) hydrogel and hyaluronic acid-gelatin (HG) hydrogel, to construct functional liver 3D organoids. Both of the studied hydrogels supported 3D spheroid organization of human liver progenitor HepaRG cells and their functional polarization. The 3D culture systems promoted hepatic differentiation of progenitor cells faster than the standard 2D culture. However, the 3D hydrogels did not enhance hepatocyte-like properties if the HepaRG cells were pre-differentiated to hepatocyte-like cells in advance. Subsequently, we showed that NFC hydrogel culture can be combined with high-resolution imaging since the intact spheroids can be enzymatically released from the matrix. This was not possible with the HG hydrogel. We demonstrated that silica bioreplication preserved the 3D spheroid structure with its fine details and cellular antigens and allowed detailed morphological analysis of the spheroids cultured in NFC hydrogel.

Next, we developed a xeno-free matrix for hepatic specification of human pluripotent stem cell-derived definite endoderm (DE) cells using a three-step approach. We first proved our hypothesis that a liver progenitor-like matrix, HepaRG-derived acellular matrix (ACM), supports hepatic lineage differentiation of DE cells. Then, we characterized the ECM proteins secreted by HepaRG cells, and finally we showed that the identified proteins, laminin-511 and laminin-521, can replicate the effect of HepaRG-ACM. The human pluripotent stem cell-derived hepatic cells expressed mature hepatocyte-like functions but the phenotype of the cells was eventually closer to fetal hepatocytes than mature cells. Thus, hepatic maturation should be further studied. In conclusion, this thesis describes new biomaterials for hepatic differentiation, a protocol to form 3D spheroids and to transfer intact spheroids to high-resolution imaging, and that the described three-step approach can guide the identification of new defined matrices.

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Helsinki, April 2016

Liisa Kanninen

Contents

List of original publications

This thesis is based on the following publications:

I Malinen MM, **Kanninen L**, Corlu A, Isoniemi H, Lou Y-R, Yliperttula M, Urtti A. Differentiation of liver progenitor cell line to functional organotypic cultures in nanofibrillar cellulose and hyalyronan-gelatin hydrogels. Biomaterials 35: 5110-5121, 2014.

II Lou Y-R*, **Kanninen L***, Kaehr B, Towson J.L, Niklander J, Harjumäki R, Brinker C.J, Yliperttula M. Silica bioreplication preserves three-dimensional spheroid structures of human pluripotent stem cells and HepG2 cells. Scientific reports 5: 1-9, 2015. *Equal contribution

III **Kanninen L**, Porola P, Niklander J, Malinen MM, Corlu A, Guguen-Guillouzo C, Urtti A, Yliperttula ML, Lou Y-R. Hepatic differentiation of human pluripotent stem cells on human liver progenitor HepaRG-derived acellular matrix. Experimental Cell Research 341: 207-217, 2016.

IV **Kanninen L**, Harjumäki R, Peltoniemi P, Porola P, Niklander J, Smutný T, Urtti A, Yliperttula M, Lou Y-R. Laminin-511 and laminin-521 based matrices for efficient hepatic specification of human pluripotent stem cells. Manuscript.

The publications are referred to in the text by their roman numerals (I-IV). Reprinted with the permission of the publishers.

Author's contribution

Publication I
The author helped to design the experiments, performed 2D and 3D cell culturing, phase contrast microscopy, alamarBlue viability assay, CYP3A4 activity, CYP3A4 induction, and whole mount immunostaining together with M.Sc. (later Ph.D.) Melina Malinen. M.Sc. Malinen performed live/dead viability assay, RT-PCR, genomic DNA quantification, immunohistochemistry, functional polarity studies, and analyzed the data. The author commented on the paper.

Publication II
The author designed the experiments with Ph.D. Yan-Ru Lou and the co-authors. The author performed HepG2 cell culturing and whole mount immunostaining. The phase contrast microscopy, immunohistochemistry, enzymatic removal of the hydrogels, silica bioreplication, and calcination were done together with co-authors. Flow cytometry of 3D cultured stem cells was done together with undergraduate (later M.Sc.) Riina Harjumäki. The scanning electron microscopy was carried out by Ph.D. Bryan Kaehr. The stem cell cultures were performed by Ph.D. Lou, undergraduate Johanna Niklander, and Ms. Harjumäki. The author analyzed the data with co-authors and wrote the paper with Ph.D. Lou.

Publication III
The experiments were designed by the author and Ph.D. Lou. The author carried out the cell culture experiments with WA07 and iPS(IMR90)-4 cell lines and the assays related to their analysis with the help of Ms. Niklander and Ph.D. Lou. M.Sc. (later Ph.D.) Pauliina Porola performed H9-GFP cell cultures and the related analysis with Ph.D. Lou. The author analyzed the data and wrote the paper together with Ph.D. Lou.

Publication IV
The experiments were designed by the author and Ph.D. Lou. The cell culturing and analysis of H9-GFP cell line was done by undergraduate (later M.Sc.) Pasi Peltoniemi and Ph.D Lou. The characterization of the HepaRG-ACM by conventional PCR was done by Ms. Porola, Mr. Peltoniemi and Ph.D. Lou and by immunostaining by the author, Mr. Peltoniemi and Ph.D. Lou. The cell culturing of WA07 and iPS(IMR90)-4 cell lines and the assays related to their analysis were performed with the help of co-authors. The author analyzed the data and wrote the paper together with Ph.D. Lou.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
ACM	Acellular matrix
ActA	Activin A
AAT	Alpha-1 antitrypsin
AFP	Alpha-fetoprotein
ALB	Albumin
BMP	Bone morphogenetic protein
CK	Cytokeratin
CV	Central vein
CXCR-4	Chemokine receptor type 4
CYP	Cytochrome P450
DPBS	Dulbecco's modified phosphate salt buffer
DE	Definite endoderm
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EHS	Engelbreth–Holm–Swarm mouse sarcoma
F-actin	Filamentous actin
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FN	Fibronectin
EpCAM	Epithelial cell adhesion molecule
GAG	Glycosaminoglycan
GFP	Green fluorescent protein
HA	Hyaluronic acid, also known as hyaluronan
HCM	Hepatocyte culture medium
hESC	Human embryonic stem cell
HG	Hyaluronan-gelatin
HGF	Hepatocyte growth factor
HNF	Hepatocyte nuclear factor
hPSC	Human pluripotent stem cell
hiPSC	Human induced pluripotent stem cell
ICAM	Intercellular adhesion molecule
LN	Laminin
MSC	Mesenchymal stem cell
MDR	Multidrug resistance protein
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance-associated protein
NaBut	Sodium butyrate
NCAM	Neural cell adhesion molecule
NFC	Nanofibrillar cellulose
NR1I2	Nuclear receptor subfamily 1 group I member 2, also known as pregnane X receptor (PXR)

NR3C1	Nuclear receptor subfamily 3 group C member 1, also known as glucocorticoid receptor (GR)
OCT4	Octamer-binding transcription factor 4
OSM	Oncostatin M
PCL	Poly-ε-caprolactone
PEG	Polyethylene glycol
PEGDA	Polyethylene glycol diacrylate
PFA	Paraformaldehyde
PT	Portal triad
qPCR	Quantitative polymerase chain reaction
RGDS	Arginine-glycine-asparagine-serine
rhEGF	Recombinant human epidermal growth factor
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
SBR	Silica bioreplication
SEM	Scanning electron microscopy
SSEA	Stage-specific embryonic antigen
TMOS	Tetraethyl orthosilicate
Wnt	Wingless type

1 Introduction

The approximated cost of bringing a new molecular entity, a novel drug, to a market is 1.8 billion US dollars, and is constantly rising (Paul et al. 2010). Only over 10 years ago the respective price was estimated to be 0.8 billion US dollars (Dickson and Gagnon 2004). The high costs are primarily due to high failure related to unaccepted efficacy (56%) and toxicity in human (28%) (Arrowsmith and Miller 2013). Currently less than one in ten drugs that enter into the clinical phase will eventually get market approval (M. Hay et al. 2014). Additionally, withdrawals from the market are still seen: 19 drugs were withdrawn in the EU during 2002-2011 (McNaughton et al. 2014). For economic and regulatory reasons, the researchers in the academia and industry are evermore looking for tools to better predict the effects of new molecules in humans (LeCluyse et al. 2012). Importantly, in 21% of the cases hepatic disorders was the reason for withdrawn drugs in the EU in 2002-2011 (McNaughton et al. 2014).

Currently, there are satisfactory pharmacokinetic in vitro models to study induction and inhibition of liver enzymes and many assays have been proposed for studying hepatotoxicity (LeCluyse et al. 2012). However, there is an urgent need for advanced physiologically-relevant models to characterize biotransformation pathways. Hepatocytes, the main cell type of the liver, are essential components in these in vitro models. Subcellular systems such as liver microsomes can be exploited for metabolic profiling but only living cells can replicate all the metabolic processes occurring in vivo (Guillouzo and Guguen-Guillouzo 2008; A.P. Li 2007). Viable hepatocytes can be isolated from human liver, and they continue to serve the golden standard cell type for in vitro tests even though there are certain limitations in their use (Guillouzo and Guguen-Guillouzo 2008; FDA 2012). One of the reasons hampering their use is their limited life-span in culture. To overcome this, significant improvements in maintaining their phenotype have been achieved when using in vivo mimicking culture matrices (Bissell et al. 1987; Michalopoulos and Pitot 1975; Uygun et al. 2010; Sellaro et al. 2010). Alternative source for obtaining viable human hepatocytes are human pluripotent stem cells (hPSC). Both human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC) have the ability to form any cell type of the human body (Thomson et al. 1998; Yu et al. 2007; K. Takahashi et al. 2007) and thus they are broadly studied for various biomedical applications, as well as for drug testing.

The concept of culturing cells was established over 100 years ago but the currently used culture techniques are not yet ideal. Cell culturing can be seen to have started in 1885 when cells were kept alive outside a body for a few days (Wall 2015). In 1907 cells were successfully grown in a laboratory for the first time (Harrison et al. 1907). Thereafter, numerous cell culture systems, reagents, and devices have been reported. Still, cell cultures are routinely performed with very simple techniques in two-dimensional (2D) configuration (Breslin and O'Driscoll 2013). Standard 2D monolayer cultures on flat, typically plastic surfaces do not simulate the complexity found in in vivo tissue as cell-matrix and multidimensional cell-cell interactions are missing (Owen and Shoichet 2010; Breslin and O'Driscoll 2013). Mimicking the cells' natural microenvironment, extracellular matrix (ECM), in vitro with biomaterials has been shown to be a successful strategy to improve tissue-like functions of the

cultured cells (Tibbitt and Anseth 2009; Breslin and O'Driscoll 2013; Owen and Shoichet 2010). Development of bioinspired materials has two approaches. The materials are either extracted or derived from nature and then modified, or the idea for a new material is from nature but the product is manufactured synthetically (Smitthipong et al. 2014). Thus, bioinspired materials can be classified as materials originating from nature or materials mimicking the nature. Developing liver mimicking culture systems for hepatocytes should start by learning from the liver.

The shifting trend from the standard 2D cultures to more advanced systems, often using the third culture dimension, challenges the basic analysis techniques. In more complex culture formats visualization of the sample or data collection can be problematic. For example, in microfluidic devices the cell number and the culture volume can be so low that detection of an analyte can cause issues, or in case of three-dimensional (3D) culture system, the matrix or a thick sample itself can hinder microscoping of the specimen (Pampaloni et al. 2007; Esch et al. 2015). Consequently, it is important that the innovations in analysis techniques keep up with the development of advanced culture systems.

This thesis describes first structure, functions, development, and ECM proteins of the liver and reviews matrix-based cell culture systems for hepatocytes. After the literature review, the aims and materials and methods of this thesis are introduced. Finally, the obtained results are briefly presented and the findings are discussed.

2 Review of the literature

2.1 Liver tissue environment

During the last decades hepatic research has significantly advanced and increased the knowledge in liver structure, function, and hepatic lineage maturation (Abdel-Misih and Bloomston 2010; Si-Tayeb et al. 2010a; L. Zhang et al. 2008). New molecular biology technologies have provided deeper understanding in regulation of gene expression, proliferation, cell cycle, and DNA repair in hepatocytes (Si-Tayeb et al. 2010a). This chapter gives an overview of the liver anatomy and function, development, describes recently proposed hepatic lineage maturation process, and illustrates the extracellular matrix chemistry in the liver.

2.1.1 Liver structure and function

In adults, liver is the largest gland and it weighs approximately 1.5 kg comprising two to three percent of body weight (Si-Tayeb et al. 2010a). The liver has various functions which are strictly dependent on correct liver tissue architecture (Figure 1A). A liver lobule is the basic architectural unit consisting of plates of hepatocytes with thickness of one to two cells (Schiff, et al. 2011; Si-Tayeb et al. 2010a). The hexagonal-shaped lobule is lined by a portal triad vessels composed of portal vein, bile duct, and hepatic artery (Figure 1B, D). The portal vein and hepatic artery supply blood to the lobule from each of the lobule's six corners and a network of sinusoidal capillaries carry the oxygen and nutrients to a central vein in the middle of the lobule. Indeed, a highly vascularized liver receives more blood than any other organ in the body (Abdel-Misih and Bloomston 2010). The periportal area close to the portal triad is also called zone 1 and the pericentral area next to the central vein is called zone 3.

Hepatocytes are the major cell type in the liver representing approximately 80% of the liver volume and 60% of all liver cells (Malarkey et al. 2005). These polyhedral-shaped cells are highly polarized, having a specialized canalicular region at their apical membrane and plenty of microvilli on the basolateral surface towards the space of Disse, the area between the hepatocytes and sinusoidal endothelial cells (Schiff et al. 2011). The canalicular regions of two neighboring hepatocytes are connected with tight junctions and form bile canaliculi that drains to the bile duct. Hepatocytes are responsible for most of the liver's biosynthesis and biomolecules' storage functions (Juza and Pauli 2014). They exhibit functional gradients over the lobule from zone 1 to zone 3 (Malarkey et al. 2005) (Figure 1C). Hepatocytes in zone 1, also called periportal hepatocytes, receive abundant blood supply rich in oxygen and nutrients and thus show higher activity in gluconeogenesis, amino acid catabolism, ureagenesis, cholesterol synthesis, and bile acid secretion compared to the cells close to the central vein, also called centrilobular cells (Malarkey et al. 2005; Schiff et al. 2011). In zone 3, the hepatocytes are responsible for glycolysis, lipogenesis, and biotransformation of endogenous and exogenous compounds.

In addition to hepatocytes, at least 14 other cells types can be found in a normal liver (Malarkey et al. 2005). Out of these, cholangiocytes, endothelial and sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells are closely cooperating with hepatocytes (Si-Tayeb et al. 2010a). Cholangiocytes, also called biliary epithelial cells, transport bile and maintain its pH (Tietz and Larusso 2006). Kupffer cells are liver macrophages with immunological and phagocytic functions and reside in the sinusoidal vessels (Malarkey et al. 2005; Schiff et al. 2011; Si-Tayeb et al. 2010a). Hepatic stellate cells, located in the spaces of Disse, are normally quiescent but when activated by various cytokines they function as the principal hepatic fibroblasts (Malarkey et al. 2005; Schiff et al. 2011; Martinez-Hernandez and Amenta 1995).

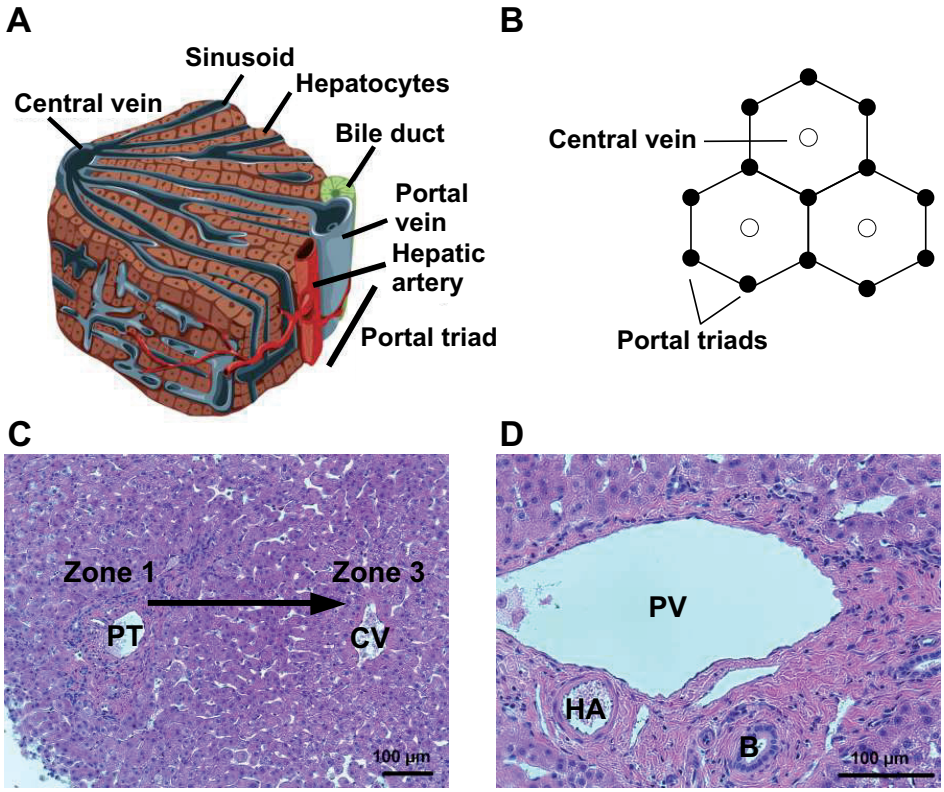


Figure 1 *Architecture of the liver. A) The main cell type of the liver are hepatocytes which are organized in plates between portal triad and central vein. B) A liver lobule, the smallest architectural unit of the liver, is lined by portal triads. C) The hepatocytes exhibit functional gradients over the lobule from portal triad (PT), zone 1, to central vein (CV), zone 3. D) The portal triad is composed of portal vein (PV), hepatic artery (HA), and bile duct (B). Picture A is modified from Si-Tayeb et al. 2010a, picture B is inspired by LeCluyse et al. 2012, and pictures C and D are from hematoxylin and eosin stained human liver sections (Lou et al. unpublished).*

2.1.2 Embryonic liver development and intrahepatic lineage maturation

Processes occurring during liver development can be categorized into two main chains of events: differentiation of all the cell types in liver from their embryonic progenitors and the arrangement of the derived cells into highly organized structures (Zong and Friedman 2014). In human, gastrulation takes place on approximately day 16 of gestation, after which the embryo is composed of ectodermal, mesodermal and endodermal germ layers. Liver epithelial cells, hepatocytes and cholangiocytes, are derived from the endoderm which also gives rise to pancreas, lung, thyroid, and gastrointestinal tract. The next step in liver development is hepatic specification during which developing cardiac mesoderm and the septum transversum mesenchyme secrete inductive signals, fibroblast growth factor (FGF) and bone morphogenic proteins (BMPs), to endoderm cells that differentiate to hepatoblasts (Gualdi et al. 1996; Zong and Friedman 2014).

The formed hepatoblasts are proliferating and form a liver bud, anatomical outgrowth from the ventral wall of the foregut endoderm (Zhao and Duncan 2005). Immediately after the bud being formed, endothelial cells or angioblasts envelop the bud and play important role in the bud's expansion and separate it from the septum transversum mesenchyme (Matsumoto et al 2001; Zhao and Duncan 2005). Next, the hepatoblasts begin to invade into surrounding septum transversum mesenchyme as cords, and simultaneously, the hepatic vasculature is being developed. The hepatoblast population is rapidly proliferating and as a bipotent cell type they differentiate into hepatocytic and cholangiocytic lineages (Schiff et al. 2011; Cardinale et al. 2011). In concert, other liver cell types are being differentiated, biliary tract is developed, and the ECM is formed (Zhao and Duncan 2005).

Multiple transcription factors, including hepatocyte nuclear factors (HNFs), have shown to be crucial for the hepatocyte differentiation (Zhao and Duncan 2005). In addition, oncostatin M (OSM), secreted by hematopoietic cells, is essential in controlling the late stage hepatocyte differentiation. The maturation of functional hepatocytes is gradual and also continues after birth. During fetal and neonatal periods, the metabolizing enzyme and transporter expressions occur in several patterns and vary for each enzyme subfamily and isoform (Moscovitz and Aleksunes 2013). The ECM proteins present in the liver are discussed below in chapter 2.1.3. At birth, the liver comprises approximately four percent of the newborn's body weight (Suchy 2014).

In a healthy adult liver, hepatocytes are proliferatively quiescent and their estimated life-span is over one year (Roskams 2006; Sell 2001). However, in the case of partial hepatectomy, surgical removal of liver mass, or in acute injury the lost liver mass is rapidly replaced although the native liver architecture is not reconstituted (Fausto and Campbell 2003). The liver has enormous regenerative capacity but results from studies on how liver regeneration occurs have been controversial. It has been shown that after a partial hepatectomy, hepatocytes resting at G0 phase re-enter the cell-cycle and start to replicate (Taub 2004). However, after toxin-mediated injuries hepatocytes have decreased capacity to proliferate. Some of the findings support the assumption that liver stem/progenitor cells (LPCs) participate in repopulating the liver after a severe injury in concert with proliferating hepatocytes (Forbes et al. 2002). In addition, a recent study reported that hepatocytes can be converted into cholangiocytes in vivo which might play a role in restoring functions and architecture

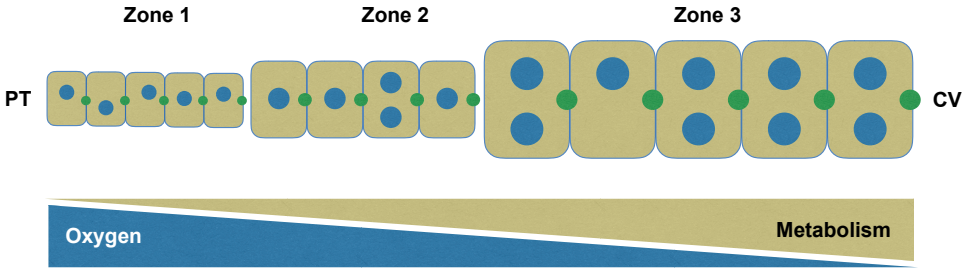
of the liver after injuries (Yanger et al. 2013).

The research on liver stem cells does not have explicit history. The early observations and speculations on LPCs were made before the 19th century and the characterization of oval cells, small epithelial cells with oval nuclei, in the 1950s started their broad examination (Fausto and Campbell 2003; Farber 1956). LPCs are classified as cells that have the ability to differentiate into hepatocytes and cholangiocytes during liver regeneration and cellular turnover (Tanimizu and Mitaka 2014). Even though the role and origin of LPCs is still not unambiguous their importance in liver biology has been proven (Forbes et al. 2002). The LPCs have been suggested to derive from periductular cells, hematopoietic stem cells, or the quiescent hepatocytes themselves have been suggested to be the stem cells of the liver as their differentiation can be activated (Fausto and Campbell 2003; Sell 2001; Y. Zhang et al. 2003). Later, Reid and co-workers have reported that both hepatic stem cells and hepatic progenitors are found in liver of all donor ages (L. Zhang et al. 2008; Turner et al. 2011; Cardinale et al. 2011).

The stem cells are located in a unique microenvironment called a niche which regulates self-renewal and differentiation of the stem cells (Scadden 2006). In pediatric and adult livers, the hepatic stem cell niches are found in canals of Hering and within the biliary tree (Theise et al. 1999; Kuwahara et al. 2008; Turner et al. 2011). It has been recently shown that peribiliary glands throughout the biliary tree host multipotent stem/progenitor cells which give rise to cholangiocytes, hepatocytes and pancreatic committed progenitors (Cardinale et al. 2011; Y. Wang et al. 2013). These biliary tree stem/progenitor cells express typical endodermal markers but only low-levels or no lineage markers of liver or endocrine pancreas (Cardinale et al. 2011).

Whether the liver is a classical stem cell lineage system or not is a divisive question in the field of hepatology (Sigal et al. 1992; Fausto and Campbell 2003). Intrahepatic lineage maturation, as suggested by Reid and co-workers, consists of eight stages proceeding from zone 1 to zone 3 in the liver lobule (Turner et al. 2011; Furth et al. 2013) (Figure 2). Multipotent hepatic stem cells represent approximately 0.5-2% of the liver cells in donors of all ages (Turner et al. 2011). The hepatic stem cells co-express epithelial cell adhesion molecule (EpCAM), neural adhesion molecule (NCAM), cytokeratin-19 (CK-19), low levels of albumin (ALB) and very low levels or no alpha-fetoprotein (AFP) (L. Zhang et al. 2008; Furth et al. 2013; Schmelzer et al. 2006). In the next stage, the hepatic stem cells differentiate into hepatoblasts which can give rise both to hepatocytic and cholangiocytic lineages. Hepatoblasts express intercellular adhesion molecule-1, (ICAM-1), EpCAM, early cytochrome P450s, and are highly positive for AFP (Turner et al. 2011; Furth et al. 2013; Schmelzer et al. 2006).

Next, the hepatoblasts are committed to hepatic progenitors which, according to this classification, are unipotent cells and can differentiate only to hepatocytes (Turner et al. 2011). Hepatic progenitors express ALB but lack CK-19 and AFP (L. Zhang et al. 2008; Furth et al. 2013). In the liver lobule, they are located in the beginning of hepatocyte plates. During the later stages of lineage maturation the hepatocytes grow in size, become binucleated, and express mature liver markers such as CYP3A4, glutathione S-transferases, and high levels of ALB (Turner et al. 2011; Furth et al. 2013). Finally, the apoptotic hepatocytes at zone 3 have been reported to produce hepatocyte growth factor that simulates the expansion of stem cells and/or progenitors which works as positive loop signaling.



Lineage stage (viable cells)	1	2	3	4	5	6	7
Parenchymal cells	Hepatic stem cells	Hepato-blasts	Comitted progenitors	Diploid adult hepatocytes			Tetraploid pericentral hepatocytes
Cell size	7-10 μm	10-12 μm	12-15 μm	15-20 μm	22-25 μm	25-30 μm	>30 μm
Cell growth	Maximum (complete cell division)			Intermediate (complete cell division)		Negligble	
Gene expression	NCAM EpCAM CK-19 AFP-	ICAM CYP3A7 AFP+++ ALB+	ALB++ AFP- Glycogen	<<< Gradient >>>		ALB+++ CYP3A4 Glutathione S-transferases	

Figure 2 *Hepatic lineage maturation from hepatic stem cells to apoptotic hepatocytes is suggested to consist of eight steps (Turner et al. 2011; Furth et al. 2013). The liver lobule exhibits structural and functional gradients from zone 1 to zone 3 (*L. Zhang et al. 2008; *Y. Wang et al. 2011; Turner et al. 2011; Furth et al. 2013). AFP, alpha-fetoprotein; ALB, albumin; CK-19, cytokeratin-19; CV, central vein; CYP, cytochrome P450 enzyme; EpCAM, epithelial cell adhesion molecule; ICAM-1, intercellular adhesion molecule-1; NCAM, neural adhesion molecule; PT, portal triad. Figure is modified from Turner et al. 2011 and LeCluyse et al. 2012.*

2.1.3 Extracellular matrix chemistry

In liver, hepatocytes are in a continuous intereaction with an ECM. The ECM is a mixture of molecules which by their structure and function provide essential cues for cell proliferation, migration and differentiation, and maintenance of tissue homeostasis (Faulk et al. 2014). In turn, dysfunction of ECM dynamics leads to unregulated cell proliferation and differentiation causing severe pathological events such as fibrosis and cancer (Rozario and DeSimone 2010; Lu et al. 2011). The secretion of the ECM starts already at the embryonic stage and its remodeling is an important mechanism by which tissue formation is regulated (Rozario and DeSimone 2010; Lu et al. 2011). The ECM is modified and degraded by enzymes secreted by the cells. The most important enzymes in ECM remodeling are matrix metalloproteinases (Lu et al. 2011).

ECM proteins are functionally diverse; both rigid, elastic, wet, and sticky proteins are needed in the tissue formation and maintenance (Mecham 2001). Biochemically, ECM components can be divided into proteins, proteoglycans, and glycoproteins (Rozario and DeSimone 2010; Lu et al. 2011). Fibrillar collagens and elastin form fibrils and determine the viscoelasticity and tensile strength of the tissue. Fibronectin and laminins contribute as building blocks of the matrix network and connecting proteins. ECM occupies only a very limited part of a healthy liver and is restricted to portal triads, sinusoids, and central veins (Bedossa and Paradis 2003). In the plates of hepatocytes, the ECM is located in spaces of Disse (Martinez-Hernandez and Amenta 1995).

The most abundant ECM proteins the liver are collagens which also are the most frequently found proteins in a human body compromising approximately 30% of the total protein mass (Bedossa and Paradis 2003; Ricard-Blum 2011). Out of the described 28 members of the collagen family, fibrillar collagen, types I, III, and V, and basement membrane collagen, types IV and VI, have been described in the liver (Y. Wang et al. 2011; Martinez-Hernandez and Amenta 1995). In addition to collagens, other matrix components found in the liver are glycoproteins, including laminins, fibronectin, and tenascin, and proteoglycans, such as hyaluronic acid, also called as hyaluronan, heparan, and chondroitin sulfate (Bedossa and Paradis 2003). Even though several groups have examined the liver ECM proteins, more studies are still needed to understand their relative quantities in different locations.

Laminins (LNs) are present predominantly in the basement membrane in most tissues in human and they bind to cell surface via integrin and nonintegrin receptors (Durbeej 2010; Malinda and Kleinman 1996). These multidomain heterotrimers are named on the basis of their α, β, and γ chains (Aumailley et al. 2005) and different subtypes can serve distinct biological functions (Colognato and Yurchenco 2000). By now, 18 laminin isoforms have been described in the literature (Durbeej 2010). Out of these, at least LN-211/221, LN-411/421, and LN-511/521 are found in adult human liver (Kikkawa et al. 2008; Liétard et al. 1998). According to Kikkawa and co-workers, LN-511 and LN-521 are the main laminin isoforms in the liver (2008). Presence of LN-111 in the adult liver is uncertain. Expression of laminin α1 chain mRNA in human liver has been reported (Liétard et al. 1998) but in other studies the α1 chain protein was not found (Virtanen et al. 2000; Kikkawa et al. 2008). In general, distribution of laminin isoforms in the human liver has not yet been broadly studied and thus a complete picture on their localization can not be made.

Fibronectin is a multidomain protein that binds to cell surface receptors, collagens, and other fibronectin molecules (Schwarzbauer and DeSimone 2011). Fibronectin is expressed in most human tissues (Stenman 1978), and it is abundant in liver as well (Martinez-Hernandez and Amenta 1995). Proteoglycans are a heterogenous group of macomolecules composing of glycosaminoglycans (GAGs), linear polysaccharides, bound to a core protein. Proteoglycans are categorized based on their GAG chain and size. Degree of sulfation of GAGs and proteoglycans is believed to play an important role in intahepatic differentiation processes (Y. Wang et al. 2010).

The ECM chemistry changes during the development, and in the adult liver expression of the matrix proteins vary between the zones. (McClelland et al. 2008; Y. Wang et al. 2010). In periportal areas, zone 1, the matrix is composed of laminins, collagen types I, III, IV, and V, fibronectin, hyalyronan, entactin, perlecan, and

chondroitin sulfate proteoglycans as shown in animal and human studies (Kikkawa et al. 2008; Seebacher et al. 1997; McClelland et al. 2008; Turner et al. 2011; Y. Wang et al. 2010; Mazza et al. 2015; Roskams et al. 1995). Kikkawa and co-workers (2008) reported that LN-511 is abundant in the bile duct where fibronectin expression has been reported to be low or absent (Hahn et al. 1980). Fibronectin, in turn, is the most abundant ECM protein in the space of Disse (Martinez-Hernandez and Amenta 1995). Other ECM components localized in spaces of Disse include all types of collagen found in the liver and perlecan (Martinez-Hernandez and Amenta 1995; Y. Wang et al. 2011; Mazza et al. 2015). Laminin is not present in the sinusoids of a healthy liver (Martinez-Hernandez and Amenta 1995; Kikkawa et al. 2008). Elastin is expressed in the whole liver lobule (Y. Wang et al. 2010; Turner et al. 2011; Van Eyken et al. 1990)

The matrix close to the central vein, zone 3, contains collagens type I, IV, and VI and a weak expression of collagen type III, fibronectin, syndecans, and highly sulfated heparan proteoglycans (McClelland et al. 2008; Y. Wang et al. 2011; Mazza et al. 2015). Zone 3 matrix differs from typical epithelial basement membrane by almost total absence of laminins and entactin, but contains large amounts of tenascin (Van Eyken et al. 1990; Y. Wang et al. 2010; Turner et al. 2011). Low or absent expression of laminin isoforms in zone 3 was confirmed by Kikkawa et al. (2008).

The matrix chemistry within intrahepatic stem cell niche is only partially characterized (Furth et al. 2013). The suggested ECM components include hyalyronan, collagen type III, α6β4 integrin-binding form of laminin, and chondroitin sulfate proteoglycan with minimal sulfation (Furth et al. 2013; Seebacher et al. 1997; L. Zhang et al. 2008; Y. Wang et al. 2010; Turner et al. 2011). As stem cells migrate from the niche, the surrounding GAGs and more extensively sulfated proteoglycans together with growth factors direct the differentiation process (Y. Wang et al. 2010). The microenvironment of hepatoblasts already differs from the stem cell niche consisting collagen types III, IV and V, α3β1 integrin-binding form of laminin, normally sulfated chondroitin sulfate proteoglycan, and heparan sulfate proteoglycans as proposed by Y. Wang and co-workers (2010).

2.2. Liver cells sources for in vitro culture

Human liver cell cultures have multiple applications both as in vitro test systems and in tissue engineering. Of the liver cells, hepatocytes are needed in drug development for metabolic profiling of drug candidates (FDA 2012). Primary hepatocytes serve as the gold standard for in vitro drug biotransformation and liver toxicity testing even though their limitations in terms of availability, rapid loss of metabolizing capacity, and batch-to-batch variation are well recognized (LeCluyse and Alexandre 2010). Contradictory to liver tissue, the isolated primary hepatocytes have low proliferation capacity in vitro which hampers their use (Fausto and Campbell 2003; Ramboer et al. 2014). After recognizing that hepatic functions of primary hepatocytes decrease rapidly during culture (Bissell 1973) numerous culture improvements by using various matrices, devices, and flow-based systems have been described (Bissell et al. 1987; Michalopoulos and Pitot 1975; Uygun et al. 2010; Sellaro et al. 2010; C. Lin et al. 2015).

Hepatocyte cell lines can be obtained from tumors or by oncogenic immortalization and thus have an unlimited lifespan (Guillouzo and Guguen-Guillouzo 2008; Rodríguez-Antona et al. 2002). Compared to primary hepatocytes, hepatic cell lines exhibit a more stable phenotype, are easily available, and they can be genetically manipulated (Gomez-Lechon et al. 2010). However, their major limitation is their relatively weak metabolizing capacity. Only a few hepatic cell lines express some liver-specific functions (Gomez-Lechon et al. 2010; Guillouzo and Guguen-Guillouzo 2008).

In addition to primary isolations and immortalized cell lines, hepatocyte-like cells can be obtained from PSCs. In 1998 Thomson and co-workers established the first hESC lines. Cells derived from the inner cell mass of a blastocyst maintained capacity to self-renew and their pluripotency, capability to form all three germ layers, when expanded in vitro (Thomson et al. 1998). Only less than ten years later, a method for reprogramming pluripotent stem cells from somatic cells was described (K. Takahashi and Yamanaka 2006; Yu et al. 2007; K. Takahashi et al. 2007). These cells, named as induced pluripotent stem cells, were produced by delivering key transcription factors related to pluripotency via viral transduction into the cells in vitro. Thereafter, hiPSCs have been reprogrammed from various somatic cell types and by several alternative methods such as non-integrating vectors, small molecules, and microRNA (Okita et al. 2008; Shi et al. 2008; Anokye-Danso et al. 2011). Generally, hiPSCs exhibit similar properties to those of hESCs but do not share the same ethical concerns. As the hPSCs have an ability to form all cell types of an adult body they are highly interesting sources of cells for various human cell-based applications including disease modelling, drug screening, and cell therapies.

Hepatic differentiation of the hPSCs has already been broadly studied (Cai et al. 2007; Duan et al. 2010; Hay et al. 2008; Si-Tayeb et al. 2010b). Still, obtaining fully mature, in vivo counterparts mimicking hepatocytes from hPSCs is challenging and none of the current protocols entirely fulfil this task. Alternative stem cell sources for hepatic differentiation are hepatic stem cells and mesenchymal stem cells (MSC) (X. Zhang and Dong 2015; Y. Wang et al. 2011). In addition, direct reprogramming of fibroblasts to hepatocytes has been recently described (Huang et al. 2014).

2.3 Matrix-guided liver cell cultures

It has been stated that hepatocytes lose their phenotypic functions once isolated from liver because the cell-matrix interactions are disrupted (Kikkawa et al. 2011). Liver environment can be simulated in vitro by using several approaches. Liver tissue can be decellularized and used as a native scaffold for reseeding cells in vitro. ECM proteins, individually or in combinations, provide cues from the liver matrix to cultured cells. Alternatively, the liver microenvironment can be mimicked with biologically inactive biomaterials to resemble mechanical properties of the liver. This chapter describes how different matrices, both 2D and 3D systems, have been used to culture human liver cells, including primary isolations and liver cell lines, or facilitate the hepatic differentiation of stem cells in vitro. The materials discussed are categorized into three groups: liver tissue-derived matrices, liver ECM-based matrices, and artificial liver mimicking matrices. Due to the high number of published matrices this review can only give examples from each matrix category.

The non-matrix-based culturing techniques, such as suspension cultures or culture models built only on co-culture setup or flow systems, are not included in this review. Figure 3 gives an overview of the used matrix-guided liver cell culture approaches.

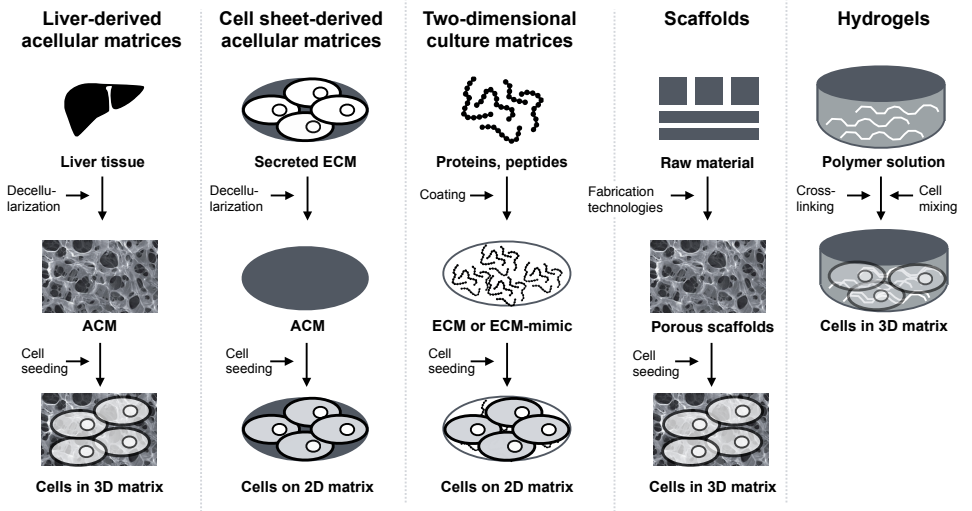


Figure 3 *Different approaches to mimic liver tissue in a dish. Liver-derived acellular matrices (ACM) have native liver structure and matrix chemistry. Cell sheet–derived ACMs can be made from cell lines. Two-dimensional (2D) culture can be performed on liver extracellular proteins (ECM) or synthetic polymers. Liver structure or stiffness can be resembled with scaffolds made from natural or synthetic biomaterials. Hepatic cells can be embedded in hydrogels, which mimic liver stiffness or matrix chemistry, to form three-dimensional (3D) spheroids. Picture is inspired by Chan and Leong 2008.*

2.3.1 Liver-derived biomaterials

Native liver tissue can be used for in vitro cell culturing after decellularization, a process which aims to remove all cellular materials from the tissue. The obtained acellular matrices (ACM) can be used as such or they can be further processed into powders and gels (Uygun et al. 2010; Y. Wang et al. 2011; Sellaro et al. 2010). After first being described in 1948, techniques for decellularization and reseeding the cells have greatly improved and currently ACMs are promising culture materials for many biomedical applications (Hinderer et al. 2016).

2.3.1.1. Whole liver acellular matrices

Decellularized whole organs are the most natural, in vivo simulating scaffolds since tissue macroarchitecture and matrix microarchitecture can be maintained after the decellularization process (Hoshiba et al. 2010). The structure, chemical composition and functions of a matrix are tissue specific but not species specific (Y. Wang et al. 2011). Though, differences in ECM component quantities between

species have been reported (Dahms et al. 1998). Animal-derived liver ACMs can be used for culturing human cells because ECM proteins are well-conserved among species and xenogeneic matrices are tolerated (Hoshiba et al. 2010).

Both whole liver and liver slices have been used to create liver structure mimicking cell culture scaffolds. The aim in tissue decellularization is to remove a whole cell population and to restore the complete tissue matrix without affecting its 3D structure, surface topology, and biological activity (Faulk et al. 2014). However, it is accepted that removal of cells from their integrin-bound anchors and intercellular adhesion sites while maintaining surface topography and resident ligands of the ECM intact is demanding (Badylak et al. 2012).

The decellularization can be performed by using enzymatic, physical, chemical and ionic methods (Badylak et al. 2012). In case of whole liver, the decellularization is preferably done by perfusion via the organ's vasculature, recent innovation that preserves the 3D structure of an organ entirely (He and Callanan 2013). The liver ACM slices have been prepared by decellularizing liver tissue sheets or sectioning the decellularized whole liver (P. Lin et al. 2004; Y. Wang et al. 2011). Before reseeding the cells into acellular liver the matrix has to be sterilized which might have a harmful effect on the mechanical properties of the scaffold (Badylak et al. 2015). The sterilization techniques include gamma irradiation, electron beam irradiation, and treatment with acid or ethylene oxide (Badylak et al. 2015; Badylak 2002; Uygun et al. 2010).

The recently described decellularization by perfusion is an attractive method to produce bioarticifial liver scaffolds. Wang et al. (2011) reported a four-step perfusion decellularization which maintains the collagen content high and the remaining growth factors and cytokines were at physiological level. Uygun and co-workers (2010) demonstrated the decullarization of rat and bovine livers by portal vein perfusion with increasing concentrations of sodium dodecyl sulfate (SDS) and sterilized with peracetatic acid. After decellularization, collagen types I and IV, fibronectin and laminins, were maintained. The acellular liver scaffold was then reseeded with rat primary hepatocytes, which showed similar ALB protein expression compared to normal liver. However, ALB and urea production were at the similar level as in the cells cultured in between collagen layers, called as sandwich culture, a much simpler technique compared to liver ACM. The concept of culturing primary hepatocytes in whole liver ACM has also been proven with mouse hepatocytes in rat liver ACM (Soto-Gutierrez et al. 2011).

Wang et al. (2011) used slices made from rat liver ACM for differentiating human hepatic stem cells to mature liver cells. Decellularization was performed with four-step protocol by portal vein perfusion including delipidation, washing step with high salt concentration to maintain the insoluble collagens, eliminating nucleic acids with nucleases, and finally washing the matrix with cell culture medium to remove salts and detergents and to equilibrate the ECM components. After freezing, the tissue matrix was sectioned, sterilized and placed to cell culture plates. Gene and protein expression data and functionality studies showed that hepatic stem cells were able to differentiate to adult liver cell types and liver specific functions were higher compared to the cells cultured on collagen type I matrix. Rat and human primary hepatocytes have also been cultured on liver ACM slices prepared from porcine tissue sheets (P. Lin et al. 2004; Lang et al. 2011). Recently, Mazza et al. (2015) described the first decellularization of a human liver. By repopulating

cubes cut from the liver ACM with three liver cell types they showed that the ACM supported homing of the different cell types into their natural locations. Liver-like functions of the recellularized ACM were not examined.

The acellular scaffolds are promising for regenerative medicine, especially for end-stage organ failures (Badylak et al. 2012). So far, transplantation of recellularized liver ACM has been tested only in animal studies (Uygun et al. 2010; Barakat et al. 2012; Mazza et al. 2015). To scale up the whole liver ACM approach, to achieve human-sized organ, researchers have engineered scaffolds from porcine and human livers (Baptista et al. 2011; Barakat et al. 2012; Mazza et al. 2015). In vitro models based on liver ACM cultures could provide a deep understanding of the biotransformation routes and mechanisms of a drug candidate. However, they will not be applicable for high-throughput drug testing, at least in the near future, due to demanding decellularization and repopulation processes using perfusion systems.

2.3.1.2 Modified liver acellular matrices

Liver ACMs can be further processed to create culture systems with less batch-to-batch variation compared to native whole tissue ACM. Already in 1980, fibrils obtained from homogenized and decellularized rat liver was used for long-term culturing of rat hepatocytes (Rojkind 1980). After that, liver ACMs have been used to create liver ECM mimicking gels and powders both for 2D and 3D cell culturing (J. S. Lee et al. 2014; Sellaro et al. 2010; Y. Wang et al. 2011).

Sellaro et al. (2010) prepared liver ACM gel from porcine liver to culture human primary hepatocytes. The hepatocytes were cultured in a sandwich configuration in between liver ACM gel layers. ALB secretion remained stable for 10 days in the cells cultured in a liver ACM gel system. However, the liver-like functions of the cells cultured in ACM gel were not improved compared to Matrigel (described in chapter 2.3.2.2) sandwich culture. Wang et al. (2011) reported exceptionally long functional stability of human primary hepatocytes cultured on pulverized bovine liver ACM. Decellularized liver matrix was milled with the presence of liquid nitrogen, mixed with culture medium and dispersed to culture wells. The human hepatocytes on ACM powder were functional for eight weeks while the cells cultured on collagen type I started to die after three weeks. A 3D hydrogel derived from rat liver ACM has been used to culture rat primary hepatocytes (J. S. Lee et al. 2014). Lyophilized ACM containing collage type I, laminins, and fibronectin was cross-linked by mixing with PBS and incubated in neutral conditions at 37°C and used to encapsulate the cells. The hepatocytes were viable for a longer period of time in 3D ACM-hydrogel culture compared to 3D collagen type I hydrogel. The cells secreted ALB and urea over one week but other hepatic functions in vitro were not characterized.

Liver ACM-derived gel has also been shown to support hepatic differentiation of mouse adipose-derived MSCs (X. Zhang and Dong 2015). The cells were plated on an ACM gel made from pulverized rat liver ACM and cultured with and without the presence of hepatic differentiation inducing reagents. Compared to other tested coating agents, collagen, fibronectin and Matrigel, the liver ACM gel supported the hepatic differentiation best. The cells treated with inducing agents showed higher hepatic functions than the non-treated cells as shown by the secretion of ALB and urea. However, the culture of stem cells without soluble factors showed that the

culture matrix alone supported induction of the MSCs towards hepatic lineage.

Compared to the whole liver ACM, the advantage of the modified liver ACM culture systems is their flexibility and possibility to decrease batch-to-batch variation by pooling several tissues together. However, the 3D structure of native liver tissue cannot be resembled with processed ACM culture systems. Nonetheless, the cells embedded in suspended or gelified ACMs can be easily transplanted in vivo with minimally invasive techniques offering a promising tool for tissue engineering (Badylak et al. 2015).

2.3.2 Liver ECM component-based matrices

The chemistry of liver tissue can be mimicked by using ECM proteins in culture systems. The ECM proteins have been used as culture substrata for liver cells since the 1970s when hepatocytes were cultured on collagen membranes (Michalopoulos and Pitot 1975). Thereafter, ECM proteins, mixtures or individual components, have been used as 2D matrices and materials for creating 3D culture systems. The ECM component-based matrices are introduced in the three following chapters: cell sheet-derived acellular matrices, ECM protein mixtures, and defined ECM proteins.

2.3.2.1 Cell sheet-derived acellular matrices

Compared to whole organ ACMs, decellularized cultured cells offer a simpler approach to mimic liver environment. Cultured cells do not require ethical permission and their decellularization can be performed without sophisticated devices. In addition, the production of cell line-derived ACMs is more straightforward compared to whole organs as the sterilization step can be avoided when performing the cultures in the same cell culture facility. The choice of the cell line for ACM construction is based on the desired ECM protein or combination of proteins secreted by the cells. The cell sheet ACMs have been developed from liver cells but also from other tissue types and tumors (Herrema et al. 2006; Vuoristo et al. 2013). The decellularization of cultured cells has been performed with distilled water or alkaline solution with or without detergent (Herrema et al. 2006; H. Takashi 2007; Vuoristo et al. 2013). The ACM can be characterized at the protein level with immunofluorescence, or at the structural level with transmission electron microscope. However, the structural studies of the cell sheet ACMs are seldom performed.

Takahashi H. et al. (2007) prepared ACM from immortalized alveolar type II epithelial SV40-T2 cells cultured on collagen type I and Matrigel. Mouse primary hepatocytes attached to the ACM similarly as to Matrigel but secreted more ALB on the ACM than on Matrigel. The authors claimed that the SV40-T2-ACM is superior to Matrigel because the matrix contains LN-511/521. Alveolar type II epithelial cells are known to secrete laminin a5 chain (Pierce et al. 1998) but its expression in the SV40-T2-ACM remains unclear as its characterization was not published by Takahashi's group (H. Takahashi et al. 2007). Another group used HEK293 cells overexpressing LN-511 in a similar kind of culture system as Takahashi et al. to differentiate mouse ES cells to hepatocytes (Shiraki et al. 2011). Human ES cells seeded on LN-511-ACM increased their ALB secretion during the culture period

confirming their hepatic differentiation. However, the expression level of ALB mRNA was much lower than that in adult liver. The derived hepatic cells were metabolically active as shown by induction of CYP3A4 enzyme activity. LN-111 and LN-521 expressing human choriocarcinoma cell line-derived ACM has been shown to support hepatic differentiation of hESCs and hiPSCs (Vuoristo et al. 2013). The hPSCs were plated on top of ACM derived from JAR cells cultured on gelatin and differentiated towards hepatic cells with stepwise growth factor treatment. The derived cells expressed human ALB in gene and protein level similarly to the cells cultured on Matrigel.

Cell sheet-derived ACMs are relatively fast, easy, and cost-efficient matrices to produce. The transfected cell lines offer possibility to create culture matrices with specific ECM protein composition mimicking a certain developmental phase or a region in the liver. However, the cell sheet-derived ACMs fail to mimic the native liver structure which can be restored in whole liver ACMs.

2.3.2.2 ECM protein mixtures

Matrigel, protein extract from Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells, is the most widely used ECM protein mixture for culturing different cell types in vitro (Kleinman and Martin 2005). The EHS cells secrete ECM proteins abundantly and commercial Matrigel mainly contains laminin-111 (c. 60%), collagen type IV (c. 30%), and entactin (c. 8%) together with numerous other proteins and several growth factors (BD Biosciences 2011; Hughes et al. 2010; Streuli et al. 1995). Matrigel can be applied in 3D culturing to embed the cells inside the hydrogel or in 2D cell culturing as a thin coating substrate.

At the end of the 1980s Bissel and co-workers discovered that Matrigel supports the maintenance of primary hepatocytes for a longer period of time than collagen type I as shown by ALB secretion of the cells (Bissell et al. 1987). Thereafter, numerous cell cultures have been performed on Matrigel coating. In addition to its use as a coating substratum, Matrigel can be applied on top of the cell monolayer to maintain or improve the morphology, polarity and maturation of the hepatocytes (Gross-Steinmeyer et al. 2005; Page et al. 2007; Bachour-El Azzi et al. 2015). Page et al. (2007) showed that Matrigel overlay increased the expression of ALB, transferrin, and transthyretin in human hepatocytes from four donors compared to cells in a control culture system without an overlay. Matrigel overlay had an inducing effect on drug metabolizing enzyme expression, especially CYP2B6 and glutathione transferases, in human primary hepatocytes (Gross-Steinmeyer et al. 2005). Compared to the conventional culture system, primary human hepatocytes formed more elongated bile canalicular network when overlaid with Matrigel as shown by phase contrast microscopy and fluorescence staining of filamentous actin (Bachour-El Azzi et al. 2015). In addition, the activity of influx transporter Na+-dependent taurocholate co-transporting polypeptide was significantly higher in the cells in Matrigel overlay culture compared to conventional culture.

In addition to primary hepatocytes, Matrigel has been commonly applied for culturing liver cell lines and stem cells for hepatic differentiation (Ordovás et al. 2013; Ramaiahgari et al. 2014; Molina-Jimenez et al. 2012; Hay et al. 2008). Liver Huh-7 cell line formed functional 3D spheroids when embedded in

Matrigel (Molina-Jimenez et al. 2012). Transporter protein MRP2 was localized at the apical membrane of the 3D Huh-7 spheroids and the spheroids secreted 5-chloromethylfluorescein di-acetate (CMFDA) into bile duct-like constructs. Ramaiahgari et al. (2014) showed that Matrigel culture also supports long-term 3D culture of human hepatocarcinoma HepG2 cells. During one month in culture, HepG2 spheroids showed upregulation in ALB secretion, formation of bile duct-like structures and functional biliary transport. The gene expression of metabolic enzymes was higher in 3D cultured cells than in 2D culture. In addition, the enzyme activities of CYP2C9, CYP3A4, and CYP2D6 were increased in 3D compared to 2D. The maintenance of hPSCs is commonly done on Matrigel coating. Thus, many researchers have described hepatic differentiation of hPSCs on Matrigel (Hay et al. 2008; Si-Tayeb et al. 2010b; Hannan et al. 2013). Indeed, hepatic differentiation on Matrigel can be regarded as a standard culture condition to which many new culture systems are being compared. In conclusion, Matrigel provides both ECM proteins and growth factors (growth factor reduced product also on the market) to cell culture models. However, one of the drawbacks of Matrigel is undefined components in the product leading to possible variations between independent cell culture experiments (De Bartolo and Bader 2013).

Another described ECM protein mixture applied in liver cell culturing, Adipogel, is extracted from conditioned media of cultured 3T3-L1 murine preadipocytes (Sharma et al. 2010; Sharma et al. 2011). Adipogel contains ECM proteins collagen type IV, laminins, HA, and fibronectin and numerous growth factors such as hepatocyte growth factor and vascular endothelial growth factor (Sharma et al. 2010). Sharma et al. (2011) showed that rat hepatocytes overlaid with Adipogel secreted over two-times more ALB compared to cells in collagen sandwich culture. However, the urea production of the cells was at a similar level.

Similarly to Matrigel, the composition of Adipogel has not been completely characterized but it offers a new, cost-effective ECM matrix for in vitro hepatocyte cultures (Sharma et al. 2010). One major drawback of both Matrigel and Adipogel is their origin and thus the cells cultured on these animal-derived matrices cannot be taken into clinical applications.

2.3.2.3 Defined ECM proteins

Individual ECM proteins can be extracted from animals or human or produced either in eukaryotic and prokaryotic expression systems or in transgenic organisms (Ruggiero and Koch 2008). Using one ECM protein at a time makes it possible to study the specific effect of the protein to cell functionality. In addition, ECM proteins are usually cost-effective culture formats and their use does not require sophisticated laboratory devices. This chapter introduces how collagens, laminins, fibronectin, vitronectin, and hyalyronic acid have been applied in liver cell cultures.

In the 1970s, Michalopoulos and Pitot successfully applied collagen membranes in culturing primary hepatocytes (Michalopoulos and Pitot 1975). Thereafter, numerous collagen-based matrices have been described. The adhesion of hepatocytes to several types of collagen has been proven but collagen type I is most used for cell culturing (Rubin et al. 1981). McCelland et al. (2008) showed that collagen types III and IV, expressed in the zone 1 of the liver lobule, facilitated cell proliferation, while

collagen type I, a zone 3 matrix protein, elicited commitment to hepatic progenitors.

It was soon noticed that monolayer culture of primary hepatocytes on a single collagen type I layer does not support long-term viability or cell functionality (Bissell et al. 1987). Dunn et al. (1989) showed that double collagen layer, below and above the cultured cells, prolongs the time of ALB secretion of rat hepatocytes. Currently, collagen type I sandwich culture is one of the standard culture systems for primary hepatocytes (LeCluyse et al. 2004). Collagen type I has been applied in hepatic stem cell differentiation as 2D coating substratum or as 3D hydrogel (X. Zhang and Dong 2015; Baharvand et al. 2006). Baharvand et al. (2006) examined hepatic differentiation of hESCs in 2D and 3D matrices made from collagen type I. They noticed that secretion of AFP and urea in 3D cultured cells was higher than those in 2D culture. However, the ALB secretion in 2D and 3D cultures were at a similar level. Commercial 3D collagen type I matrix, RAFT™ 3D Cell Culture System, was shown to promote maturation of hiPSC-derived pre-differentiated hepatic cells (Gieseck et al. 2014). Interestingly, the maturation was evident only if the pre-seeded hepatic cells were seeded to 3D matrix in aggregates. When seeded to 3D collagen as single cells the ALB secretion was dropped, indicating the importance of cell-cell contact for mature hepatocytes.

Recently laminins have been actively studied for culturing hepatic cells. The use of cell sheet-derived ACMs containing laminins have been described above. Recombinant laminins LN-111 and LN-521 have been successfully used for culturing hepatic cells (Cameron et al. 2015; Takayama et al. 2013; Takayama et al. 2014). Human ES cells, plated on LN-521 or a mixture of LN-521 and LN-111, were differentiated to functional hepatocyte-like cells (Cameron et al. 2015). The derived cells on LN matrices showed higher CYP1A2 and CYP3A enzyme activity compared to cells cultured on Matrigel. The CYP3A enzyme activity in cells on LN-521 and LN-111 were highest from day 20 to day 24 after which the activity dramatically decreased. Cameron et al. also showed that the derived hepatocyte-like cells had functional efflux transport as evidenced by a vectorial transport assay with 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDFDA). Takayama et al. (2013) tested four different laminins for culturing hESC and hiPSC-derived hepatoblasts. The attachment of pre-differentiated hepatoblasts to LN-111 was approximately three-times higher than that to LN-211, LN-411, and LN-511. The group showed that they were able to maintain the hepatoblasts cultured on LN-111 up to 15 passages as shown by stable AFP protein expression in the entire cell population. In addition, 10-times passaged hepatoblasts differentiated better to hepatocytes compared to hepatoblasts without passaging as shown by higher expression of CYP enzymes, and higher ALB and urea secretion. Later, the same group differentiated human liver-derived iPSCs to hepatocyte-like cells in a similar culture setting with some modifications (Takayama et al. 2014). Again, the cells passaged at the hepatoblast stage showed higher expression of typical hepatocyte markers and secreted more ALB and urea compared to the cells without passaging. Kikkawa et al. (2011) showed that peptides derived from laminin chains are also able to support the attachment of rat hepatocytes. Even though the gene expression of hepatic markers were not improved compared to Matrigel, the synthetic peptides offer a defined and xeno-free culture matrix.

As fibronectin is expressed in most human tissues, it has been widely used as a coating material for culturing different cell types (Stenman 1978). Indeed, it was

stated over 30 years ago that fibronectin is a relatively nonspecific cell attachment protein (Carlsson et al. 1981; Donato et al. 1995). In liver, fibronectin is expressed in the whole lobule but most abundantly in zone 3 (Y. Wang et al. 2011). Several studies have shown that fibronectin supports the attachment and viability of hepatocytes (Carlsson et al. 1981). However, it has been revealed that fibronectin did not promote the attachment of hepatic progenitors, isolated from human fetal liver, but instead caused rapid cell death (McClelland et al. 2008).

Cell adhesion glycoprotein vitronectin has been shown to support the hepatic differentiation of hPSCs (Nagaoka et al. 2015). Nagaoka et al. combined a fragment of vitronectin containing RGD (arginine-glycine-asparagine) peptide into immunoglobulin G antibody and plated hPSCs on this matrix, called as R-Fc. The derived hepatic cells were positive for HNF4A as shown by immunofluorescence and had a capacity for activity uptake of indocyanide green, a specific function of hepatic cells. However, the expression of AFP revealed that the cells were not fully mature. The R-Fc matrix could offer an alternative, highly defined coating substrate to Matrigel. However, based on the gene expression profile Matrigel-cultured cells expressed more mature hepatocyte markers compared to R-Fc-cultured cells.

Hyaluronic acid is a nonsulfated glycosaminoglycan abundantly expressed in stem cell niches and thus it is an attractive culture matrix for hepatic stem cells (Turner et al. 2013). HA hydrogel has been shown to maintain the phenotype of hepatic stem cells and moreover, support the hepatic differentiation of this cell type (Lozoya et al. 2011; Turner et al. 2013). Lozoya et al. (2011) showed that hepatic stem cells isolated from fetal human liver can be differentiated towards hepatic progenitors and hepatocytes when cultured in 3D HA hydrogel. They noticed that stiffness of the hydrogel plays an important role in the hepatic differentiation. The hydrogel with shear modulus, a measure of gel stiffness, of 73-220 Pa guided the hepatic differentiation the best.

In summary, the defined ECM proteins are easy to use and their commercial availability is high. Some of the commercial ECM proteins are xeno-free and GMP-grade which enable the transfer of cultured cells to clinical use. However, the single ECM protein-based cell culture matrices do not mimic the complexity of matrix chemistry found in liver.

2.3.3 Artificial liver mimicking matrices

Numerous biomaterials that do not originate from liver tissue or do not contain any liver matrix components have been applied in liver cell culturing either as 2D matrices or 3D hydrogels and scaffolds. The biomaterials described below are categorized as naturally-derived and synthetically-derived biomaterials depending on their source of polymer. The combinations of two natural biomaterials or a natural and a synthetic biomaterial, so called hybrid biomaterials, are introduced as well. Liver ACMs and ECM component-based matrices also belong to the classification of natural biomaterials but are already introduced in chapters 2.3.1 and 2.3.2.

2.3.3.1 Natural biomaterials

The source of natural biomaterials ranges from plants to mammals and the naturally-derived polymers include proteins such as silk, fibrin, and gelatin, or polysaccharides, for example, alginate, chitosan, and cellulose (Allen et al. 2015; Bhattacharya et al. 2012). As any other cell culture matrices, naturally-derived biomaterials can be used to create both 2D and 3D culture systems. This chapter gives examples of the use of alginate, chitosan, silk, and cellulose in liver cell cultures. Other described natural biomaterials for liver cell culturing include materials such as fibrin and gelatin (Chinzei et al. 2002; X. Wang et al. 2006; Bruns et al. 2005).

Alginates are linear polysaccharides commonly obtained from brown seaweed (Lee and Mooney 2001). These biodegradable polymers are one of the most used biomaterials in tissue engineering (N. Lin et al. 2010; Rowley et al. 1999). Alginate scaffolds have been shown to support culture of various types of liver cells. However, 2D alginate films seem to inhibit the attachment of hepatocytes which is most likely caused by the lack of interaction between the material and cells (Glicklis et al. 2000). Adult rat hepatocytes showed stable ALB secretion in 3D alginate scaffold for seven days while rat newborn liver cells continued producing ALB for over 40 days (Dvir-Ginzberg et al. 2003; Dvir-Ginzberg et al. 2008). Indeed, 3D alginate scaffold, cross-linked with D-gluconic acid by freeze-drying, supported the maturation of cells isolated from newborn rat liver cells and the formed spheroids secreted approximately 3000-times more ALB than the cells cultured in a similar scaffold fabricated from collagen (Dvir-Ginzberg et al. 2008). Lin et al. (2010) proved that 3D alginate scaffold is also suitable for hepatic differentiation. They differentiated rat MSCs into hepatocyte-like cells in calcium cross-linked alginate scaffold. The hepatic differentiation was confirmed by increased ALB and urea production over the culture period. However, the differentiation was not performed in standard 2D culture so it remains unclear if the 3D configuration in alginate scaffold improved the hepatic maturation.

Chitosan is derived from chitin containing shells such as crabs and shrimps after purification and enzymatic or chemical conversion steps from chitin to chitosan (Cheung et al. 2015). Li et al. (2003) compared hepatic functions of rat primary hepatocytes plated on a chitosan monolayer and in a 3D porous chitosan scaffold. After one week of culture, ALB secretion in 3D cultured cells was approximately two-times higher than in 2D cultured cells, while there was no difference in urea production. In addition to primary hepatocytes, chitosan matrices have been used to culture HepG2 cells (Verma et al. 2007) and to differentiate human MSCs towards hepatocyte-like cells (Cheng et al. 2012).

Fibrillar biomaterials such as silk and nanocellulose have been shown to elicit the growth of liver cell lines (Kundu et al. 2013; Bhattacharya et al. 2012). Kundu et al. (2013) demonstrated HepG2 cell culture in 3D silk scaffold. Silk fibroins, produced by silkworms, were fabricated into 3D scaffold by molds and lyophilized. HepG2 cells were viable in the silk scaffold for 21 days but the group did not characterize the hepatic functions of the cells. Wood-derived nanofibrillar cellulose (NFC) is obtained from plant cell walls by enzymatic hydrolysis and mechanical shearing (Pääkkö et al. 2007). NFC hydrogel is formed at low fiber concentrations and has been shown to support the culture of HepG2 and HepaRG cells (Bhattacharya et al. 2012). HepaRG cells were viable after one month of 3D culture and secreted ALB at increasing trend

during the culture therefore demonstrating the maturation towards hepatocytes. Cellulose has also been formulated into macroporous scaffolds to culture HepG2/C3A and primary hepatocytes (Yue at al. 2010; Nugraha et al. 2011).

2.3.3.2 Synthetic biomaterials

In principle, synthetic chemistry allows creating cell culture matrices with nearly any shape, stiffness, porosity, or charge. Synthetic biomaterials can provide a more controlled and reproducible microenvironment for cultured cells compared to the naturally-derived biomaterials (Lutolf and Hubbell 2005). Synthetic polymers are often functionalized with peptides or growth factors to increase their biochemical activity as they do not offer biological cues for cells by themselves.

Polyurethanes are regarded as one of the most biocompatible materials (Zia et al. 2015). Hay et al. (2011) screened 380 polyurethane and polyacrylate polymers to identify suitable synthetic matrices for 2D culturing of hPSC-derived hepatocyte-like cells. Polyurethane 134 polymer was found to support the attachment of pre-differentiated hepatocytes and maintain their liver-specific functions. Compared to Matrigel culture, the cells cultured on polyurethane 134 produced more fibrinogen, transthyretin, and fibronectin, and their CYP1A2 activity was approximately six-times higher.

3D scaffolds have been developed from numerous synthetic polymers. Adwan et al. (2013) described that highly porous 3D scaffold made from polyhedral oligomeric silsesquioxane and poly-ε-caprolactone (PCL) supported hepatic functions of HepG2 cells for two weeks as shown by their urea secretion. Lee and co-workers (2009) showed that the size of HepG2 spheroids can be precisely controlled in an inverted colloidal crystal scaffold made from polyacrylamide. The spheroids with diameter of 50 μm showed higher ALB production over the culture period compared to larger spheroids. However, ALB secretion was not improved compared to 2D cultured cells. Poly-L-lactic acid mixed with polyglycolic acid scaffold was used for hepatic differentiation of mouse ESCs (Liu et al. 2010). Embryoid body-derived cells were mixed with Matrigel and cell culture medium and seeded onto scaffold. During 20 days in culture, upregulation of ALB secretion confirmed hepatic maturation of the derived cells. Hepatic differentiation of mouse MSCs in electrospun polyamide fibers coated with Matrigel was demonstrated by Piryaei et al. (2011). Scaffold made from positively charged fibers induced hepatic differentiation more than the scaffold with negative charge as shown by ALB and CYP1A1 protein expression. However, there was no clear difference in urea, AFP, and ALB production between these two scaffolds.

Polyethylene glycol (PEG)-based biomaterials are widely applied in cell cultures due to their versatility (Underhill et al. 2007). PEG-biomaterials are biocompatible, hydrophilic and easily crosslinked by light (Lutolf and Hubbell 2005; Underhill et al. 2007). Rat primary hepatocytes encapsulated in PEG-hydrogel prolonged their ALB and urea secretion when the hydrogel was functionalized with adhesive peptide RGDS (arginine-glycine-asparagine-serine) (Underhill et al. 2007). Improved hepatic functions by additive RGD peptide were also shown by Itle et al. (2005) with liver cell line. PEG-diacrylate (PEGDA) hydrogel functionalized with RGD significantly improved the ALB secretion in SV-40 transformed murine hepatocytes.

Commercial peptide hydrogel, Puramatrix™, has been shown to support 3D culture of rat hepatocytes and HepG2 cells (S. Wang et al. 2008; Malinen et al. 2012). Rat hepatocytes in 3D Puramatrix secreted ALB and urea over 20 days at the same level compared to the cells cultured in collagen type I sandwich and Matrigel but exhibited approximately two-times higher CYP3A4 activity compared to collagen sandwich culture on day nine (S. Wang et al. 2008). Malinen et al. (2012) showed that HepG2 cells formed 3D spheroids with bile duct-like constructs with efflux transporters MDR1 and MRP2 localization. In addition, the functionality of hepatobiliary transporters was confirmed by demonstrating the transport of fluorescein diacetate into formed canalicular-like structures. Puramatrix can also be used in sandwich culture systems as an alternative to ECM proteins (Genové et al. 2009). Rat hepatocytes cultured in between two layers of Puramatrix secreted ALB at a similar level as the cells in standard collagen sandwich after one week culture period. However, long-term stability of hepatic functions should be further studied to reveal if the synthetic sandwich could replace traditionally used collagen.

2.3.3.3 Hybrid matrices

Hybridization of biomaterials offers more possibilities to modify the matrix characters to create a desired culture matrix. By combining a natural and a synthetic biomaterial or two or more naturally-derived polymers together the physicochemical and biological properties of a matrix can be tuned. These hybrid materials are also called bio-artificial or biosynthetic polymeric materials (Zia et al. 2015). Due to the fact that possibilities to design different types of hybrid biomaterials are nearly unlimited, many hybrid matrices have already been suggested for liver cell culturing. Thus, this chapter gives examples of the used matrices rather than a complete review.

Multiple biomaterials that do not originate from liver have been combined with ECM proteins, especially with collagen type I, to increase cell-matrix interactions. Biologically inert PCL was combined with collagen type I and polyethersulfone to achieve porous nanofiber scaffold to differentiate MSCs towards hepatocyte-like cells (Kazemnejad et al. 2009). Compared to standard 2D culture, the 3D cultured cells secreted approximately two-times more ALB indicating improved status of hepatic differentiation. Li et al. (2010) also used 3D scaffold made from polylactic-co-glycolic acid and collagen type I for hepatic differentiation of MSCs. Similarly, they showed that 3D scaffold supported the hepatic differentiation better compared to monolayer cultured as shown by ALB and urea synthesis. Collagen type I was also combined with PEGDA to fabricate inverted colloidal crystal scaffolds (M. H. Kim et al. 2015). By increasing the collagen concentration in the matrix the ALB secretion of Huh-7 cells increased. In addition to ECM proteins, polymers can be conjugated with galactose to increase cell adhesion (Yin 2003; Stampella et al. 2015). Stampella et al. (2015) demonstrated that HepG2/C3A cells secreted significantly more ALB and urea when they embedded in alginate scaffold supplemented with galactose as compared to a standard monolayer culture on cell culture plastic.

The mechanical properties of natural hydrogels can be improved by combining them with synthetic polymers. HA can be coupled with both natural and synthetic polymers. Commercially available HyStem® (previously Extracel®), HA hydrogel

crosslinked with PEGDA, and HyStem®-C, which contains additional gelatin component, are in situ gelifying hybrid hydrogels of which stiffness can be altered by changing the ratio of the kit's components (Shu et al. 2006; Prestwich et al. 2007). Rat primary hepatocytes cultured in HyStem maintained their CYP1A2 activity for 17 days, five days longer than cells cultured in standard 2D format (Prestwich et al. 2007). Lozoya et al. (2011) performed an extensive screen of different ratios of HA and PEGDA to find the optimal matrix for human hepatic stem cells. They noticed that stiffness of the HA matrix controls the differentiation to hepatoblasts and committed progenitors in vitro. The secretion of AFP, ALB and urea increased in hydrogels with lower concentration of HA suggesting hepatic lineage maturation. Also, You et al. (2013) studied the effects of hydrogel stiffness to hepatic cell functionality. Primary rat hepatocytes were plated on heparin-PEGDA matrices with stiffness range from 10 to 110 kPa. Cell attachment, morphology, ALB expression and ALB secretion were significantly higher on soft, 10 kPa, matrices compared to stiffer gels. Heparin-PEGDA hybrid matrix was also used to encapsulate rat primary hepatocytes to obtain 3D spheroids (M. Kim et al. 2010). The hepatic functions of hepatocytes were negligible when the cells were cultured in matrix containing only PEGDA while the ALB secretion in heparin-PEGDA was maintained for 20 days in culture.

Hybrid biomaterials are also used to build liver structure mimicking culture scaffolds. Jiankang et al. (2009) developed a chitosan-gelatin scaffold that resembles the architecture of liver lobule for culturing rat hepatocytes. The hepatocytes secreted ALB and urea over the studied nine days but further functional studies should be performed to estimate whether the suggested matrix could be used, for example, for drug testing. 3D printing techniques have opened new possibilities to control the organization of cells and matrices in vitro. Billiet et al. (2014) fabricated cell-laden 3D constructs by printing hydrogel and HepG2 cells. The HepG2 cells were mixed with gelatin-methacrylamide hydrogel and dispensed into a symmetrical 3D fiber network. Viability of the printed cells was high and ALB and HNF4A expressions were maintained after the printing. 3D printing offers attractive technique for culturing hepatocytes which are highly organized cells in vivo. However, more studies are needed to confirm the potential of this technique.

Finally, the most important criteria for selection of a biomaterial for a cell culture system are biocompatibility and non-toxicity. In general, the advantage of natural biomaterials is their better biocompatibility compared to synthetic materials. However, the composition of natural matrices is often poorly defined which might cause variability between individual cell culture experiments. Both naturally-derived biomaterials and synthetic biomaterials can be produced with various techniques, such as electrospinning, photolithography, and 3D printing, and their properties can be tuned by altering, for example, cross-linking density or changing the functional groups. Hybridization of different types of materials enables even more possibilities to create desired culture conditions. A drawback of many artificial liver mimicking matrices is that they do not provide biological binding sites for cells and thus the cell-material interactions are missing. Instead, some of the matrices form ionic interactions with cells, or the scaffold provides only structural support (J. Li et al. 2003; Glicklis et al. 2000).

3 Aims of the study

The hypothesis of the thesis was to show that new biomaterials for in vitro hepatic differentiation can be found by mimicking the natural ECM and by studying the natural mechanisms. To prove the hypothesis, this thesis focuses on finding, characterizing, and testing matrices for maturing human liver progenitor cells to functional hepatocytes and differentiating the hPSCs into hepatic lineage. The specific aims were:

1. To construct functional spheroids in natural and hybrid 3D hydrogels and to couple the culture format with high-resolution imaging to analyze intact spheroids (I, II).
2. To test the hypothesis that a liver progenitor-like matrix can support hepatic lineage specification of hPSC-derived DE cells (III, IV).
3. To study if characterization of ECM proteins secreted by liver progenitor cells can lead to identification of hepatic lineage stage specific biomaterials and development of defined ECM protein-based matrices (III, IV).

4 Materials and methods

The materials and methods used are briefly described in this chapter. The detailed descriptions of the experimental procedures and methods are found in the original publications I-IV. Table 1 summarizes the used methods.

Table 1 *Summary of methods and cell types used in the studies.*

Method	Cells	Publication
2D cell culturing	HepaRG, HepG2, H9-GFP, iPS(IMR90)-4, WA07	I, II, III, IV
3D cell culturing	HepaRG, HepG2, iPS(IMR90)-4, WA07	I, II
Albumin ELISA assay	H9-GFP, iPS(IMR90)-4	III, IV
Silica bioreplication	HepG2, iPS(IMR90)-4, WA07	II
Conventional PCR	HepaRG	IV
CYP activity measurement	HepaRG, iPS(IMR90)-4	I, IV
CYP induction	HepaRG, iPS(IMR90)-4	I, IV
Enzymatic degradation of hydrogel	HepG2, iPS(IMR90)-4, WA07	II
Flow cytometry	H9-GFP, iPS(IMR90)-4, WA07	II, III
Functional polarity assay	HepaRG	I
Genomic DNA quantification	HepaRG	I
Immunostaining	HepaRG, HepG2, H9-GFP, iPS(IMR90)-4, WA07	I, II, III, IV
Immunohistochemistry	HepaRG, WA07	I, II
Confocal microscopy	HepaRG, HepG2, H9-GFP, iPS(IMR90)-4, WA07	I, II, III, IV
Phase contrast microscopy	HepaRG, HepG2, H9-GFP, iPS(IMR90)-4, WA07	I, II, III, IV
qPCR	HepaRG, H9-GFP, WA07, iPS(IMR90)-4, liver tissue, primary human hepatocytes	I, III, IV
Scanning electron microscopy	HepG2, iPS(IMR90)-4, WA07	II
Viability assays	HepaRG, WA07	I, II

4.1. Cell culture matrices

4.1.1 Two-dimensional matrices

4.1.1.1 Human liver progenitor cell-derived acellular matrix

Human liver progenitor cell-based ACM was prepared from HepaRG cells. The human liver progenitor cell line HepaRG was obtained from Biopredict (Gripon et al. 2002). The cells were plated at 26,000 cells/cm^2 density and cultured for two weeks in previously prescribed culture conditions (Aninat et al. 2006; Cerec et al. 2007). The ACM was prepared with water treatment as described earlier with minor modifications and used immediately for plating the cells (Herrema et al. 2006). To characterize the matrix composition the ECM protein expression of the HepaRG cells was analyzed by conventional PCR and immunofluorescence (see chapters 4.3.2.2 and 4.3.3.2).

4.1.1.1 Extracellular matrix proteins

Tissue culture plates were coated with ECM proteins LN-511, LN-521, LN-111, collagen type I, collagen type III, fibronectin, or ECM protein mixture Matrigel. LN-511, LN-521, and fibronectin were used both individually and with seven possible combinations. Mouse LN-111 (Cultrex, 25 µg/ml) and rat tail collagen type I (Cultrex, 50 µg/ml) and human collagen type III (Sigma, 25 µg/ml) solutions were incubated in wells for two hours at RT. Human rLN-511 and rLN-521 (Biolamina, 10-20 µg/ml) and human fibronectin (Sigma-Aldrich, 25 µg/ml) were incubated for two hours at 37°C or for overnight at 4°C. Matrigel coating (BD Biosciences) was prepared according to manufacturer's instructions. The LN-511, LN521, and fibronectin solutions were collected after use, stored at -20°C, and reused up to two times.

4.1.2 Three-dimensional matrices

Plant-derived nanofibrillar cellulose hydrogel, GrowDex®, was obtained from UPM-Kymmene Corporation, Finland. The stock hydrogel, typically 1.7 wt% was diluted to working concentration of 1.0 wt% with cell culture medium by pipetting. Hyaluronan-gelatin (HG) hydrogel, Extracel® (nowadays HyStem®-C), was purchased from Glycosan Biosystems (nowadays ESI BIO), and the gel was prepared according to the manufacturer's instructions. HG hydrogel is composed of thiol-modified hyaluronic acid, thiol-modified gelatin (denatured collagen), and thiol-reactive crosslinker PEGDA.

4.2 Cell cultures

4.2.1 Human liver cell lines

Human hepatocellular carcinoma HepG2 cells (ATCC, HB-8065; Knowles et al. 1980) were maintained in either in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 1 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin or in high glucose DMEM (Gibco) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. HepaRG cells were cultured in William's E medium (Gibco) supplemented with 10% FBS, 50 IU/ml penicillin and 50 μg/ml streptomycin, 5 μg/ml insulin, 1 mM L-glutamine and 50 μM hydrocortisone.

Both HepG2 and HepaRG cells were maintained in 75 cm^2 cell culture flasks in a humidified tissue culture incubator at 37°C in 5% CO2. HepG2 cells were passaged twice a week and HepaRG cells every two weeks. To promote hepatic maturation, the HepaRG progenitor cells were cultured for two more weeks with the presence of 2% dimethyl sulfoxide (DMSO) which induces the differentiation towards hepatocyte-like cells (Gripon et al. 2002).

4.2.2 Human primary hepatocytes and liver tissue

Cryopreserved primary human hepatocytes (BD Biosciences, a 29-year-old female donor and a 13-year-old male donor) were recovered by using a cryopreserved hepatocyte purification kit (BD Biosciences) according to the manufacturer's instructions. Human liver tissue was obtained from four donors from the Transplantation and Liver Surgery Clinic (Helsinki, Finland) which 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. Both primary hepatocytes and liver tissue were used as controls in real time polymerase chain reaction (RT-PCR).

4.2.3 Human pluripotent stem cells

The hESC lines WA07 and WA09 (also known as H9) (Thomson et al. 1998) and hiPSC line iPS(IMR90)-4 (K. Takahashi et al. 2007; Yu et al. 2007) were purchased from WiCell research institute. H9 cells were genetically modified to H9-GFP cells as earlier described by us (Lou et al. 2014). The stem cells were cultured in a feeder-free system using Matrigel as a coating material (BD Biosciences). The mTeSR™1 medium (STEMCELL™ Technologies) was changed daily. Before cell passaging by using Versene 1:5000 (Invitrogen), spontaneously differentiated areas were removed by a pipette.

4.2.4 Hepatic differentiation of human pluripotent stem cells

The hPSCs were first induced to DE-like cells and then stepwise differentiated towards hepatic cells (Figure 4). Protocols for differentiation media were modified from earlier reported protocols (D'Amour et al. 2005; Hay et al. 2008; Si-Tayeb et al. 2010b; Toivonen et al. 2013). The DE cells were plated either on the HepaRG-ACM or ECM proteins. Matrigel overlay was used in the last step of the differentiation for cells cultured on HepaRG-ACM.

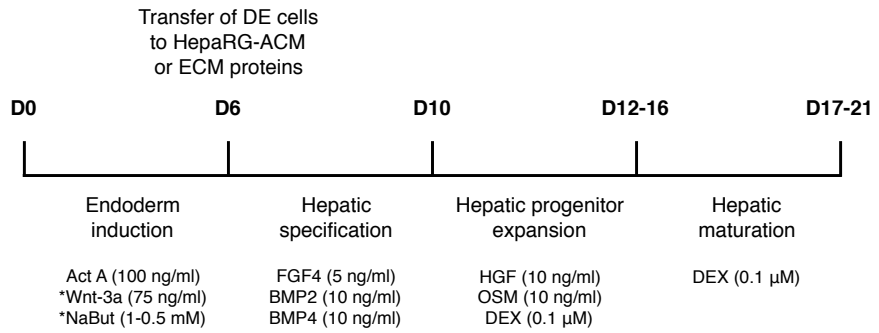


Figure 4 *Differentiation protocol of human pluripotent stem cells to hepatocyte-like cells in vitro. The stem cells were induced to definite endoderm (DE) cells with RPMI-1640 basal media (Gibco) supplemented with growth factors. After transferring the DE cells on studied matrices, the cells were cultured in Hepatocyte Culture Medium (Lonza; without rhEGF and gentamicin-amphotericin-1000) with stage specific growth factors. *Components used only with H9-GFP cells. ACM, Acellular matrix; Act A, Activin A; BMP, Bone morphogenetic protein; DEX, Dexamethasone; ECM, Extracellular matrix; FGF, Fibroblast growth factor; HGF, Hepatocyte growth factor; NaBut, Sodium butyrate; OSM, Oncostatin M; Wnt, Wingless type.*

4.2.5 Three-dimensional cell culturing

The HepG2 and HepaRG cells were first detached with 0.25% or 0.05% Trypsin-EDTA (Gibco), respectively, and the WA07 and iPS(IMR90)-4 cells with Versene 1:5000 (Invitrogen). Embedding of the cells to GrowDex hydrogel was performed as described earlier by us (Lou et al. 2014; Bhattacharya et al. 2012). Briefly, the liver cell suspension was mixed with stock GrowDex hydrogel by pipetting to achieve 1.0 wt% concentration. The stem cell colonies were gently mixed with 0.5 wt% GrowDex hydrogel. Low-retention pipette tips (TipOne®, Starlab Group) were used when handling GrowDex. The cells were encapsulated into HG hydrogel according to manufacturer's instructions.

The HepG2 cells and undifferentiated progenitor HepaRG cells were seeded to hydrogels at the density of 1.0 x 10^6 cells/ml and differentiated HepaRG cells at the density of 9.0 x 10^6 cells/ml. In 3D cultures the stem cell colony density was adjusted five-times higher than that in 2D cultures. An equal medium volume to hydrogel volume was carefully added on top of the hydrogels.

4.2.6 Enzymatic degradation of hydrogels

To release the spheroids from the 3D matrices the GrowDex hydrogel was degraded with cellulase enzyme (VTT, Turku, Finland) as earlier described (Lou et al. 2014) and HG hydrogel with 1 x collagenase/hyaluronidase (StemCell Technologies) according to the manufacturer's protocol. The intact spheroids were recovered from GrowDex by incubating them with the cellulase enzyme for 24 hours at 37°C in 5% CO2. The spheroids cultured in HG hydrogel could not be released as the enzyme digestion broke down the spheroid structure and resulted in single cell suspension.

4.3 Analysis methods

4.3.1 Viability assays

The cell viability in 3D hydrogel cultures was determined with Trypan blue exclusion test, alamarBlue reagent (Invitrogen), or live/dead viability/cytotoxicity kit (Molecular Probes) according to the manufacturers' instructions. The cells were exposed to alamarBlue reagent for three hours at 37°C in 5% CO2 and the fluorescence of medium samples was recorded with a plate reader (Varioskan Flash, Thermo Fisher Scientific). In live/dead assay the cells were treated with calcein-AM and ethidium homodimer-1 (EthD-1) and imaged with confocal microscope (see chapter 4.3.6.2).

4.3.2 Gene expression

4.3.2.1 RNA isolation and cDNA conversion

Total RNA was extracted from liver tissue with TRI reagent (Sigma Aldrich) or RNAlater (Qiagen), and from 2D and 3D cultured cells with TRIzol reagent (Invitrogen) or RNeasy Mini kit (Qiagen) according to the manufacturers' instructions. RNA concentrations were measured with a NanoDrop 2000 spectrophotometer (Thermo Fischer Scientific). The RNA was converted to cDNA with a High Capacity RNA-to-cDNA kit (Applied Biosystems) or with RevertAid H minus first strand cDNA synthesis kit (Thermo Scientific). The cDNA samples were used in conventional PCR and qPCR.

4.3.2.2 Conventional PCR

The gene expression of the ECM proteins in HepaRG cells was studied by conventional PCR with a KAPA HiFi HotStart kit (KAPA Biosystems, KK2501). The PCR cycles were performed on a DNA Engine Dyad Peltier Thermal Cycler (Bio-Rad Laboratories). The PCR cycling conditions included 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 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 a base pair ladder (O'GeneRuler™ Low Range DNA Ladder, SM1203, Fermentas).

4.3.2.3 Quantitative PCR

The cDNA samples were analyzed on a StepOnePlus Real-Time PCR System (Applied Biosystems) using Fast SYBR Green Master Mix (Applied Biosystems) or TaqMan Universal Master Mix II (Applied Biosystems). The primers were synthesized by Oligomer Oy (Helsinki, Finland). Ribosomal protein, large, P0 (RPLP0) and cyclophilin G were used as reference genes. The relative mRNA expression was calculated either by using relative standard curve or comparative C_T-experiment.

4.3.2.4 Genomic DNA quantification

The cell samples were lysed with RLT buffer (Qiagen) and genomic DNA was quantified with Quant-iT™ PicoGreen dsDNA assay kit (Molecular probes). Genomic DNA was used to determine the cell proliferation during the culture period and to normalize CYP3A4 activity and mitochondrial activity.

4.3.3 Protein expression

4.3.3.1 Flow cytometry

The 2D cultured cells were detached by Cell Dissociation Buffer (Gibco) followed by Accutase cell detachment solution (Millipore). The 3D spheroids were first recovered from NFC hydrogel with cellulase enzyme treatment (chapter 4.2.6) and then disintegrated to single cell suspension with Cell Dissociation Buffer and Accutase. Next, the cells were incubated with primary antibodies mouse anti-SSEA-4 (Developmental Studies Hybridoma Bank), anti-SSEA-3 (STEMCELL™ Technologies), or anti-CXCR-4 (R&D Systems). After washing, the cells were incubated with APC-conjugated goat anti-mouse IgG (SouthernBiotec), goat anti-rat IgM (STEMCELL™ Technologies), or goat anti-mouse IgG (Beckman Coulter). The negative control samples were treated only with the secondary antibody. The samples were analyzed on a BD LSR II flow cytometer using BD FACSDiva software. The overlay histograms were created with Flowlogic software.

4.3.3.2 Immunofluorescence and immunohistochemistry

Protein expression was analyzed by direct immunofluorescence staining from 2D and 3D cultured cells or from histological sections made from 3D spheroids. The staining of whole 3D spheroids was performed either in intact hydrogel culture or after enzymatic release in test tubes. The 2D cells were fixed with 4% paraformaldehyde (PFA) for 10-15 min and 3D spheroids for 15-30 min depending on the spheroid size. Spheroids in HG hydrogel were fixed overnight with 4% PFA. Stem cell spheroids and spheroids in HG hydrogel were embedded in paraffin and the samples were cut in 5-20µm sections. After deparafinization the sections were treated with boiling sodium citrate buffer to retrieve the antigens.

After fixation, the cell membrane was permeabilized with either 0.1% Triton X-100 or 0.5% Saponin for 10-15 min (2D cultures) or for 15-30 min (3D cultures). Next, the cells were blocked with 10% normal goat or donkey serum for one hour RT or overnight at 4°C. The primary antibodies were incubated with the cells overnight. On the following day the cells were incubated with the secondary antibodies conjugated with Alexa Fluor 594 (Invitrogen) for one to four hours (2D) or five hours (3D). Filamentous actin was stained with Alexa Fluor 594 phalloidin (Invitrogen) and cell nuclei with DAPI (Sigma-Aldrich), Hoechst 33258 (Sigma Aldrich), or SYTOX green (Invitrogen). Samples on objective glasses were mounted with ProLong® Gold antifade reagent (Invitrogen). Stained intact spheroids were placed in optical imaging microplate and protected with ProLong® Gold antifade reagent or SlowFade® Gold antifade reagent (Invitrogen) before confocal imaging (see chapter 4.3.6.2).

4.3.4 Cell functionality

4.3.4.1 Albumin ELISA assay

Secretion of human ALB in cultured cells was determined with Human Albumin ELISA Quantitation Set (Bethyl Laboratories) according to manufacturer's protocol. The ALB amount was normalized to total protein content. The cells were lysed with 1 x RIPA buffer (ThermoFisher Scientific) with 1 x protease inhibitor cocktail (Sigma Aldrich) after which the protein amount was measured with a Pierce BCA Protein Assay Kit (ThermoFisher Scientific) by following the manufacturer's instruction.

4.3.4.2 CYP3A4 activity measurement

The activity of CYP3A4 was studied with P450-Glo™ CYP3A4 assay (Promega) containing luciferin isopropyl acetate (luciferin-IPA) according to the manufacturer's protocol. The cells were exposed to luciferin-IPA for 60 min at 37 °C in 5% CO2. Luminescence was recorded with a plate reader.

4.3.4.3 CYP3A4 and CYP3A7 induction

The inducibility of CYP3A4 and CYP3A7 enzymes was studied with known inducing substrates. The cells were exposed either to dexamethasone, phenobarbital, rifampicin, or DMSO. The induction was analyzed with P450-Glo™ CYP3A4 assay (see chapter 4.3.4.2) or with qPCR (see chapter 4.3.2.3).

4.3.4.4 Functional polarity assay

Hepatobiliary transport was investigated with fluorescein diacetate (Bravo et al. 1998; Barth and Schwarz 1982). The cells were exposed to 10 μM fluorescein diacetate and the cell nuclei were stained with Draq5 (BioSatus). The cytoplasmic conversion of fluorescein diacetate to fluorescein and its export from cell cytoplasm were followed with confocal microscope.

4.3.5 Silica bioreplication

Fixed cells were incubated in a 100 mM tetraethyl orthosilicate (TMOS) solution in 1 mM hydrochloric acid overnight (2D samples; Kaehr et al. 2012) or for 24–72 hours on a shaker (3D samples) at 38°C. HepG2 spheroids in an intact HG hydrogel culture were silicified in Lab-Tek® Chamber Slide™ systems in the TMOS solution at 38°C for 72 hours. Silicified samples were sequentially washed with nano-pure water at pH 3, 1:1 water-methanol solution, and finally 100% methanol and dried in air. To study the protein expression in silicified spheroids the samples were treated with a dilute, buffered hydrofluoric acid (Transene, TIMETCH) to remove silica. The desilicified spheroids were then used for immunostaining (see chapter 3.3.3.2). Bioreplicas without the organic material were generated by calcining the silicified spheroids in air at 500°C for 16–24 hrs.

4.3.6 Imaging

4.3.6.1 Phase contrast microscopy

The cell morphology and growth were followed with phase contrast microscope (Leica DM Il LED). Pictures were captured with LAS EZ software (Leica Microsystems).

4.3.6.2 Fluorescence microscopy

Fluorescent probes were imaged with Leica TCS SP5 II HCS A confocal microscope using either HCX PL APO 20×/0.7 Imm Corr (water or glycerol) or APO 0.7 CS air objective. DAPI and Hoechst were excited with a UV (diode 405 nm/50 mW), calcein, EthD-1, and fluorescein with an argon laser (488 nm/35 mW), Alexa Fluor 594 with a DPSS (561 nm/20 mW) laser, and Draq5 with a HeNe (633 nm/12mW) laser. Emission was acquired with PMT and HyD detectors. The images were

analyzed with Imaris program (Bitplane) by creating slice, surpass, or easy 3D displays. Immunofluorescence of the H9-GFP cells and their derivatives was imaged with a Zeiss Axioplan microscope.

4.3.6.3 Scanning electron microscopy

The 2D and 3D samples were deposited onto either borosilicate cover glasses or silicon substrates and sputter-coated with Au/Pd. Scanning electron microscope (SEM) images were recorded using an FEI Quanta series scanning electron microscope.

4.3.7 Statistical analysis

Statistical significance was determined by one-way ANOVA followed by Holm-Sidak post-test using SigmaPlot software. Differences of $p < 0.05$ were considered as significant.

5 Results

The main results of this thesis are briefly presented below and discussed in chapter 6. The detailed results, including figures, are found in the original publications I-IV and in their supplements. The characterized biomaterials used as cell culture matrices for in vitro hepatic differentiation are summarized in table 2.

5.1. Three-dimensional matrices promote hepatic differentiation and NFC hydrogel enables high-resolution imaging of 3D spheroids

Our aim was to identify a simple 3D cell culture setup from which the formed 3D cell aggregates could be easily transferred for high-resolution analysis. First two different types of hydrogels, fibrillar wood-derived NFC hydrogel and a hybrid HG hydrogel, were tested to create 3D liver organotypic cultures from human liver progenitor HepaRG cells.

Both NFC and HG hydrogels supported the formation of organized multicellular aggregates, spheroids, after embedding the cells as progenitors at undifferentiated stage (I, Fig. 1 A). When the HepaRG cells were pre-differentiated to hepatocyte-like cells, they formed clearly less organized spheroids in NFC hydrogel (I, Fig. 1 B). The spheroid formation in HG hydrogel was approximately the same at both differentiation stages.

The 2D and 3D-cultured HepaRG cells were characterized in terms of their liver marker expression and hepatic functions. Compared to human liver tissue, undifferentiated HepaRG cells in all studied conditions showed similar or higher mRNA expression of hepatocyte markers, ALB, MDR1, and MRP2, after two weeks of culture (I, Fig. 4A). CYP3A4 expression in 3D-cultured spheroids was nearly at the similar level to that of human liver after one week of culture but the expression decreased that after (I, Fig. 4A). Similar expression profile in 3D-cultured spheroids was observed also with HNF4A, MDR1, MRP2, and with ALB in NFC-cultured cells (I, Fig. 4A). However, CYP3A4 activity level in 3D spheroids increased throughout the culture showing dissimilar profile to mRNA expression. CYP3A4 enzyme activity of HepRG cells embedded in NFC hydrogel increased to a significantly higher level compared to cells in HG hydrogel or in 2D culture when the cells were seeded at undifferentiated stage (I, Fig. 5 A).

When the HepaRG cells were seeded to a culture system at differentiated stage, the 2D-cultured cells showed the highest HNF4A, ALB, CYP3A4, MDR1, and MRP2 mRNA expression throughout the culture period (I, Fig. 4 B). In addition, CYP3A4 activity was the highest in the 2D culture (I, Fig 5B). However, CYP3A4 enzyme was inducible with rifampicin, phenobarbital, and DMSO in both 2D and 3D cultures (I, Supplement Fig. 1).

After two weeks in culture, the 3D spheroids had formed bile duct-like structures with apical MRP2 and MDR1 expression as observed by immunofluorescence staining (I, Fig. 6 A). The expression of these transporters were also observed in 2D cultures. The functional polarity was confirmed by following canalicular transport of

Table 2 *Properties of the cell culture matrices used in this thesis.*

	NFC hydrogel (I, II)	HG hydrogel (I, II)	HepaRG-ACM (III)	LN-111 (III)	LN-511 and LN-521 (IV)	Collagen type I (III)	Collagen type III (III)	Fibronectin (IV)	Matrigel (III)
Type of matrix in this study	3D	3D	2D	2D	2D	2D	2D	2D	2D
Origin	Wood pulp	Semi-synthetic	Human liver cell line	Murine EHS sarcoma cells	Recombinant protein	Rat tail tendons	Human placenta	Human plasma	Murine EHS sarcoma cells
Defined composition	Yes	Yes	Partially	Yes	Yes	Yes	Yes	Yes	Partially
Xeno-free	Yes	No	No	No	Yes	No	No	No	No
Commercial availability	GrowDex®, UPM-Kymmene	HyStem®-C, ESI BIO	No	Cultrex® Laminin-1, Trevigen	LN-511™, LN-521™, Biolamina	Cultrex® Collagen I, Trevigen	Sigma-Aldrich	Sigma-Aldrich	BD Biosciences
Batch-to-batch variation	Yes, fiber concentration can vary	Variations negligible[d]	Yes, protein content can vary	Not known	No	Not known	Variations in solubility[a]	Yes, protein concentration can vary	Yes, protein content can vary
Similarity to liver tissue	NFC fiber diameter similar to collagen fiber[b]	ECM chemistry (HA)	ECM chemistry	Not expres-sed in adult human liver[c]	ECM chemistry	ECM chemistry	ECM chemistry	ECM chemistry	ECM chemistry (- LN-111)
Supports hepatic differentiation of HepaRG cells	+++	++	NA	NA	NA	NA	NA	NA	NA
Supports hepatic differentiation of hPSC-derived DE cells	NA	NA	++	- No DE cell attachment	+++	- No DE cell attachment	- No DE cell attachment	++	- No DE cell attachment

ACM, acellular matrix; DE, definite endoderm; ECM, extracellular matrix; EHS, Engelbreth–Holm–Swarm, HA, hyaluronic acid; LN, laminin; NA, not analyzed in this thesis; NFC, nanofibrillar cellulose; a, Variations anticipated: solubility can vary 10% (Sigma-Aldrich's technical support 2016); b, Bhattacharya et al. 2012 and Y. Wang et al. 2011; c, Kikkawa et al. 2008; d, No variations in ECM component concentrations but degree of thiolation can vary (ESI BIO's technical support 2016)

fluorescein into formed bile canaliculi-like structures (I, Fig. 7 A). In summary, the 3D hydrogel cultures promoted faster hepatic differentiation compared to standard 2D culture but the 3D configuration did not improve the hepatocyte-like properties if the cells were differentiated before embedding.

Next, we examined coupling of the NFC and HG 3D culture systems to high-resolution imaging to study morphogenesis of 3D cultured cells. We embedded human HepG2 liver cells and human PSCs into NFC and HG hydrogels, and enzymatically degraded the matrices before analysis. However, we could not include the HG hydrogel culture in the future analysis since the hPSCs did not form spheroids in it and releasing whole HepG2 spheroids was not possible (II, Fig. 1 A; II, Supplementary Fig. 1). Instead, both HepG2 and hPSC spheroids were successfully released from the NFC hydrogel with cellulase enzyme.

We then formed stable cell-silica composites by silica bioreplication (SBR) of 3D spheroids, which enabled us to follow the growth and morphological changes during the culture. The SEM imaging of non-silicified spheroids showed deformed and collapsed structures, a dramatic difference compared to silicified spheroids (II, Fig. 2 A, B). With SBR, we noticed that HepG2 cells and hPSCs formed tight cell-cell interactions during the culture period and that cells cultured in 3D format were more round than those in 2D culture (II, Fig. 2 B).

In addition, SBR preserved fine cellular and extracellular structures as shown by dense microvilli-like formation on the cell membrane and the ECM-like network at the surface of HepG2 cells (II, Fig. 3 A, B). Cellular antigens partially remained during the SBR and were detectable by immunofluorescence. In the hPSCs, nuclear protein OCT4 was detected before and after SBR in the cell nucleus (II, Fig. 4 A, B). However, within HepG2 cells apical protein MRP2 and filamentous actin were diffused to cytoplasm during SBR (II, Fig. 4 B). In addition, we showed that all organic material of a spheroid can be removed by calcination without destroying the microstructures of 3D silica-bioreplica (II, Fig. 3 C).

In summary, this thesis increases the current knowledge on 3D liver cell culturing in hydrogels and describes a new analysis procedure to examine 3D spheroid morphology. This work supplements the earlier described NFC cell culture and analysis protocols (Bhattacharya et al. 2012; Lou et al. 2014). Taken together, NFC hydrogel is a useful culture system to produce intact spheroids for various applications and analysis methods (Fig. 5).

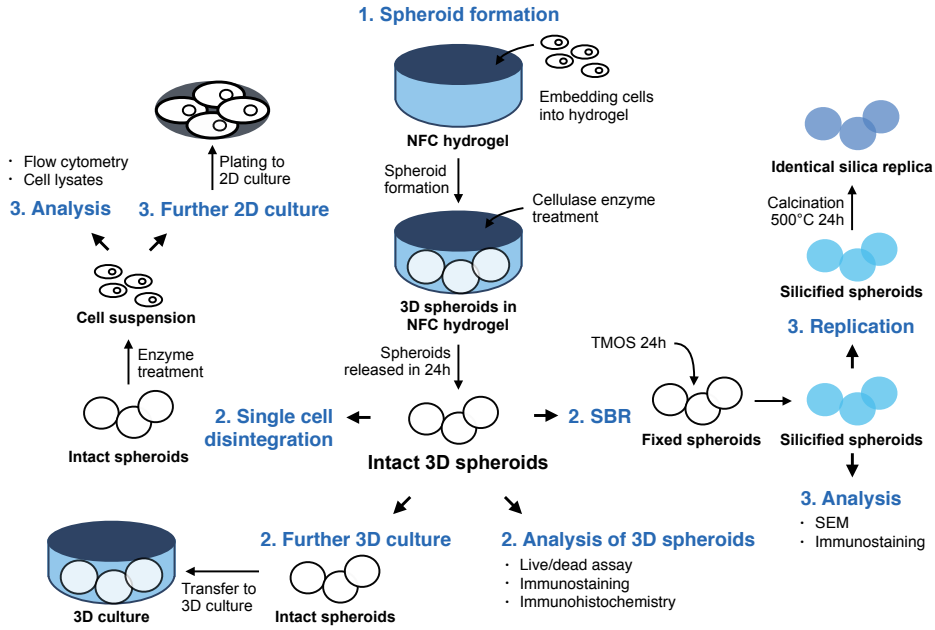


Figure 5 *Nanofibrillar cellulose (NFC) hydrogel culture can be combined with multiple analysis methods. The intact three-dimensional (3D) spheroids can be released from the hydrogel by cellulase enzyme treatment. Next, the whole spheroids can be analyzed or their structure can be stabilized by silica bioreplication (SBR). Additionally, the silicified spheroids can be calcined to create identical silica replicas of spheroids without organic material. The released intact spheroids can be transferred into further 3D culture systems. In addition, the intact spheroids can be disintegrated into cell suspension by enzyme treatment and the single cells can then be either further analyzed or transferred into two-dimensional (2D) culture format. The spheroids in NFC hydrogel culture can also be analyzed without their release by phase contrast microscope and confocal microscope. SEM = scanning electron microscopy; TMOS = tetraethyl orthosilicate.*

5.2. Laminin-511 and laminin-521-based matrices support hepatic specification of definite endoderm cells

In this project, our aim was to find a stage-specific matrix for hepatic differentiation of DE cells. To achieve the goal, we used a three-step approach in which we first tested the hypothesis (III), characterized the critical matrix components (IV), and developed a defined matrix for hepatic specification (IV).

First, we created ACM from human liver progenitors, HepaRG cell line, to test the hypothesis that human liver progenitor-like environment would assist the hepatic lineage differentiation. In addition to HepaRG-ACM, we examined other commonly used ECM protein-based matrices, including LN-111, collagen type I and III, and Matrigel. In the first step of the differentiation the hPSCs, WA07, H9-GFP, and iPS(IMR90)-4 cell lines, were induced to DE-like cells. Over 93% of the used hPSC lines expressed key pluripotent marker SSEA-3 or SSEA-4 as shown by flow cytometry analysis and based on the immunofluorescence staining the cells were

positive for OCT4 (III, Supplementary Fig. 1).

DE-inducing medium was optimized for each cell line as we noticed that similar growth factor treatment is not efficient for all the used hPSC lines. After six days of ActA, Wnt-3a, and NaBut exposure the H9-GFP-derived cells were highly positive for DE markers HNF3B and CXCR-4 (III, Fig. 1 F; III, Fig. 2). The WA07 and iPS(IMR90)-4 cells lines were induced to DE-like cells with ActA alone for six days after which over 90% and 80% of the cells, respectively, were CXCR-4 positive (III, Fig. 1 F). The HNF3B was expressed in the majority of the WA07 and iPS(IMR90)-4-derived cells (III, Fig. 2).

The derived DE cells were transferred to the studied matrices, LN-111, collagen type I and III, Matrigel, and HepaRG-ACM, and to standard cell culture plastic. Other matrices except HepaRG-ACM failed to support attachment and/or growth of DE cells (III, Supplementary Fig. 3) and they had to be excluded from the further studies.

The cells transferred to HepaRG-ACM were step-wise differentiated towards hepatocytes. The derived progenitors expressed HNF4A, CK-19, and AFP as shown by immunofluorescence staining (III, Fig. 4). In the end of the culture, the WA07-derived cells were positive for HNF4A and partially positive for ALB, CYP3A4, and AFP (III, Fig. 5). The iPS(IMR90)-4-derived cells were weakly positive for HNF4A and CYP3A4, negative for ALB, and the minority of the cells were positive for AFP (III, Fig. 5). The majority of the H9-GFP-derived hepatic cells were positive for HNF4A and partially positive for ALB and AFP.

The cultures overlaid with Matrigel showed decreased AFP and CK-19 mRNA expression (in WA07 and iPS(IMR90)-4 cell lines) and induced expression of MRP2 transporter protein (in H9-GFP cell line) at the end of the differentiation. Increased human ALB secretion over the culture period confirmed functional hepatic differentiation of H9-GFP-derived cells (III, Supplemenatary Fig. 5). Overall, the derived hepatic cells still expressed AFP indicating their immature status and the mRNA expression of mature liver markers, ALB and AAT, were greatly lower compared to human primary hepatocytes.

In the next step of this work, we characterized the ECM proteins secreted by HepaRG cells to be able to replace the ACM with defined ECM proteins. Fibronectin, laminin α2, α5, β1, β2 and γ1 chains, and collagen type IV α1, α2 and α5 chains were detected at mRNA level (IV, Supplementary Fig. 1). Fibronectin, laminin α5 chain, and collagen type IV α2 and α5 chains were also expressed at protein level (IV, Fig. 1). The result indicates 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. As collagen IV α2 and α5 chains do not form any known heterotrimers collagen matrix was not considered for the further studies.

In the final, third step, we tested if we can replicate the effect of HepaRG-ACM with the identified HepaRG matrix proteins. The ECM proteins found in human progenitor HepaRG cells, LN-511, LN-521 and fibronectin, were used as culture matrices for hPSC-derived DE cells. The hPSCs, WA07, H9-GFP and iPS(IMR90)-4, expressed the key pluripotent markers OCT4 and/or SSEA-4, and expressed HNF3B and CXCR-4 at the DE stage (IV, Supplementary Fig. 2 A, B). The DE induction was performed similarly as described above by using different growth factor cocktails for H9-GFP cells than to WA07 and iPS(IMR90)-4 cell lines. To identify the optimal culture conditions for hepatic differentiation, we screened seven different ECM

matrices composed of LN-511, LN-521, and fibronectin, tested different laminin concentrations, and optimized the seeding density of DE cells. The used laminins were xeno-free recombinant proteins and fibronectin was extracted from human plasma.

We observed that laminins alone support the hepatic specification and that fibronectin is not a vital matrix protein for the hPSC-derived DE cells. The hepatic specification was efficient on most of the seven studied matrices as shown by high expression of the hepatocyte marker HNF4A in the WA07 and iPS(IMR90)-4-derived progenitors (IV, Fig. 3). Similarly, the ALB expression in the derived hepatocyte-like cells was similar between the studied matrices as shown by immunofluorescence (IV, Fig. 4). After screening the seven matrices we did not notice any improvement in hepatic differentiation efficacy with the presence of fibronectin. Thus, the experiments were continued only with laminins.

Both hESC and iPSC-derived hepatic cells cultured on laminin matrices exhibit similar morphology to earlier described hESC-derived hepatocytes and increased ALB mRNA expression and ALB secretion confirmed their hepatic maturation (IV, Fig. 5; IV, Fig. 8; IV, Fig. 7 A; IV, Supplementary Fig. 3 A). Upregulated mRNA expression of other liver markers, AAT, CYP3A7, CYP3A4, CYP1A2, and glucocorticoid receptor, also indicated the hepatic-lineage differentiation (IV, Fig. 8). The mRNA expression of CYP1A2 and CYP3A7 in iPSC-derived cells was comparable to the primary hepatocytes (IV, Fig. 8). The hepatic cells cultured on LN-521-based matrices showed metabolic enzyme activity as shown by upregulation of CYP3A7 and CYP3A4 mRNA after dexamethasone treatment (IV, Fig. 7 B). However, the induction was not detected at the activity level as measured by CYP3A4 enzyme activity.

In summary, this thesis proves that it is possible to find new stage-specific matrices for hepatic differentiation. The work also shows that the desribed three-step approach is useful for developing defined culture matrices, and that this approach guided us to identify LN-511 and LN-521 proteins for hepatic lineage differentiation of DE cells. The workflow to develop defined ECM protein-based 2D matrices is illustrated in figure 6.

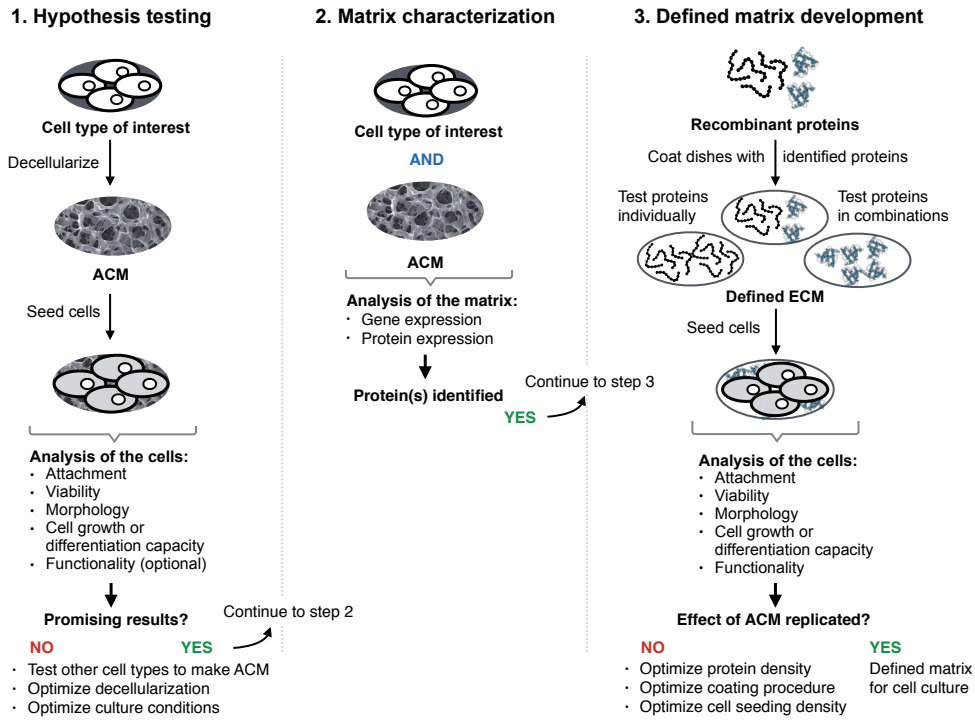


Figure 6 *Three-step workflow to develop defined matrices. In the first step, the hypothesis is tested by preparing an acellular matrix (ACM) from the cell type of interest and using the matrix for plating the cells of interest. If promising results are obtained, the extracellular matrix (ECM) proteins secreted by the cells are characterized. Once the ECM components are identified, they are tested individually and in combinations for plating the cells of interest.*

6 Discussion

There is an urgent need for functional human hepatocytes in drug development to investigate biotransformation pathways and possible hepatotoxicity of a new drug candidate (LeCluyse et al. 2012). The viable hepatocytes used in in vitro testing can be primary cells derived from human liver, regarded as a golden standard, liver cell lines, or differentiated from stem cells (Guillouzo and Guguen-Guillouzo 2008). In the liver, hepatocytes are in continuous interaction with other cells and the surrounding ECM. To create in vivo mimicking cell culture systems, the lessons need to be learnt from nature. Many researchers have shown that culturing cells with the presence of a biomaterial enables natural cell-cell and/or cell-matrix interactions, and thus, improves the viability and functionality of the cells in vitro. Indeed, in standard 2D cell culture conditions the tissue-specific structures as well as biochemical and mechanical interactions are lost (Pampaloni et al. 2007). Matrix-based culture systems for hepatocytes have been developed from natural, synthetic and hybrid biomaterials and the cells can be grown in 2D or 3D configuration. In addition to matrix-based systems, 3D liver cell cultures can be generated using bioreactors, microfluidic devices, hanging drop technique, and suspension systems with and without stirring (Tong et al. 2016; Griffith et al. 2014; Darnell et al. 2011; Y. Takahashi et al. 2015; Higuchi et al. 2016).

Development of novel liver cell culture systems and matrices should start from learning from the native liver. In tissue, the complex microenvironment provides biochemical and mechanical cues to hepatocytes, blood flow creates shear stress and the matrix chemistry and stiffness are not static (Owen and Shoichet 2010; Schoen et al. 2001; Abdel-Misih and Bloomston 2010). The in vivo characters of liver should guide the design of in vitro cell cultures. On the other hand, the aimed use of the cells, available materials and devices, as well as time and cost, also play a part in developing cell culture models. Besides the biological understanding, the development of new culture systems requires new materials and/or devices for building the models, and finally new analysis techniques for monitoring the advanced systems.

6.1 Mimicking liver tissue in a dish with bioinspired matrices

New bioinspired matrices can be generated by modifying existing natural materials or synthesizing novel materials based on the idea from nature (Smitthipong et al. 2014). Mimicking native tissue in a dish can be approached by simulating the stiffness, ECM chemistry, shear stress, or the structure of the liver. In this thesis, NFC hydrogel, HG hydrogel, HepaRG-derived ACM, LN-511, LN-521, and fibronectin were used as culture matrices for human hepatic cell lines or to differentiate hPSCs towards hepatocyte-like cells (I, II, III, IV; Table 2).

6.1.1. Three-dimensional hydrogels induce differentiation of liver cells

3D cell culture matrices were already introduced over 40 years ago (Elsdale and Bard 1972). Hydrogels are attractive biomaterials for 3D cell culturing since they can be made from a wide range of natural or synthetic polymers and their fibrous structure mimics the native ECM fibers to some extent (Tibbitt and Anseth 2009). Despite their popularity, hydrogels have not been widely used for culturing human liver progenitor HepaRG cells this far (Bhattacharya et al. 2012; Colosi et al. 2014; Dianat et al. 2014). Other described 3D culture formats for HepaRG cells include tethered spheroid culture, bioreactor, hanging-drop system, a micropatterned agarose substrate, and a suspension culture with a presence of aggregation inhibiting polymer (Z. Wang et al. 2015; Y. Takahashi et al. 2015; Darnell et al. 2011; Mercey et al. 2010; Higuchi et al. 2016). HepaRG is a bipotent cell line that can differentiate to hepatocyte-like cells and cholangiocyte-like cells (Cerec et al. 2007). Compared to other existing liver cell lines, differentiated HepaRG cells are functionally more similar to primary human hepatocytes (Lübberstedt et al. 2011; Kanebratt and Andersson 2008; Hart et al. 2010). In addition, HepaRG cells have already been used for testing acute and long-term toxicity (Mueller et al. 2014; Leite et al. 2012).

We showed that 3D hydrogel culture induced the differentiation of HepaRG progenitor cell line to organotypic 3D spheroids with functional bile duct-like structures (I). We noticed that NFC hydrogel promotes rapid differentiation of HepaRG cells as indicated by gene expression of typical liver markers. Thus, functional studies could be performed in the future after a one-week culture period to investigate if the 3D cultured cells could be used, for example, for drug testing one week earlier than the typical two-week culture time. The metabolically active organoids might serve a useful liver model in drug development for substance testing. However, the replacement of standard 2D cell cultures with 3D culturing in the pharmaceutical industry will still take time (Rimann and Graf-Hausner 2012). On the other hand, once the reproducibility and higher throughput with reasonable cost are achieved, the 3D cell culture technologies will likely become more common in drug and chemical testing.

Mechanical properties of liver tissue change during its development and vary between regions of the liver (Lozoya et al. 2011). The stiffness of the culture matrix has shown to also affect hepatic cell fate in vitro (Lozoya et al. 2011; Hamilton et al. 2001). Lozoya et al. (2011) described that stiffness of the HA hydrogel plays an important role in the hepatic differentiation. They reported that a hydrogel with a stiffness of 73-220 Pa induced the differentiation of hepatic stem cells to hepatic progenitors the best. Much stiffer HA hydrogels were suggested for culturing primary human hepatocytes (Deegan et al. 2015). Deegan et al. reported that the stiffness between 1200-4600 Pa is optimal for maintaining primary hepatocyte functions. The reported stiffness values of normal liver vary from 2 to 7 kPa (Rouvière et al. 2006; Roulot et al. 2008). We did not measure the stiffness of HG hydrogel but according to earlier reports it is below 100 Pa (Vanderhooft et al. 2009). The storage modulus of the other studied biomaterial, 1.0 wt.% NFC hydrogel, is approximately 100-200 Pa (Pääkkö et al. 2007; Harjumäki et al. unpublished). Despite the fact that the stiffness of the studied hydrogels is one magnitude less compared to those recorded from liver, the hydrogels successfully induced liver functions in HepaRG spheroids (I). However, the comparison of reported stiffness values is not fully

reliable if the analysis method is not exactly the same.

The trends in the field of hydrogel research have been shown to shift from using simple polymer networks to using smart, functional gels (Buwalda et al. 2014). Indeed, the fourth dimension, control over time, has already been introduced in cell culturing (Tibbitt and Anseth 2012). The mechanical properties of NFC hydrogel could be varied during the culture by increasing or decreasing fiber concentration, and future studies could include a careful screening of different hydrogel concentrations to analyze if the varying stiffness affects hepatic differentiation of HepaRG spheroids. It would be especially interesting to study if a stiffer NFC hydrogel than the tested 1.0 wt.% would support the maintenance of differentiated status of HepaRG cells. We observed that the expression of hepatocyte markers decreased when the cells were embedded into hydrogels at differentiated state (I). We have not studied the reason for this but it could be due to the fact that the spheroids seeded at differentiated state were less polarized than the spheroids embedded at undifferentiated state as observed by immunofluorescence and functional polarity assay. In the future, further studies with NFC could also include functionalization of the hydrogel. Dissimilarly to HG hydrogel, NFC hydrogel does not provide biochemical cues to cultured cells as it does not contain any ECM components.

In general, NFC hydrogel is an interesting new xeno-free biomaterial already tested for a few other biomedical applications including wound care and drug delivery (Kolakovic et al. 2012; Mertaniemi et al. 2016; Laurén et al. 2014). NFC hydrogel has structural similarities to ECM in liver. Cellulose nanofibers of NFC hydrogel are 20-30 nm in diameter on average and several micrometers in length, which are similar characteristics to those of native collagen fibers in the liver (Bhattacharya et al. 2012; Y. Wang et al. 2011). Compared to other commercial hydrogels, the most significant advantage of NFC hydrogel is that it can be degraded with a specific enzyme to release viable spheroids for further analysis or culture (Lou et al. 2014; discussed more in chapter 6.2). A disadvantage of the product is its tendency to adhere on plastic surfaces, and thus, special low retention pipette tips are required for its dispensing. In addition, when working with low fiber concentration, for example with 0.5 wt.% NFC, which is optimal for hPSCs (II; Lou et al. 2014), the hydrogel is very loose and changing of cell culture media is challenging.

6.1.2 Lineage-specific matrix guides efficient hepatic differentiation

Human PSCs have gained a solid foothold in basic research but also in the drug industry as they have potential to offer a limitless supply of various somatic cell types needed in drug development (Grskovic et al. 2011). Differentiation of hESCs and hiPSCs to hepatocyte-like cells has been studied extensively (Duan et al. 2010; Si-Tayeb et al. 2010b; Hay et al. 2008; Cai et al. 2007). However, the scientific community still faces the major challenge in the differentiation process; derived hepatic cells are immature and their phenotype is closer to fetal hepatocytes than mature cells (Schwartz et al. 2014; Baxter et al. 2015). In the majority of the published protocols, hPSCs are guided towards hepatocytes with growth factors through DE stage (Baxter et al. 2015; Gerbal-Chaloin et al. 2014). Indeed, during embryogenesis the cells from definitive endoderm differentiate to fully functional adult hepatocytes under specific biochemical and mechanical cues (Zaret 2001).

Growth factors commonly introduced to hPSCs to differentiate them to DE-like cells in vitro include Activin A, Wingless-related integration site 3A, and, sodium butyrate (D'Amour et al. 2005; Hay et al. 2008; Toivonen et al. 2013). At the next step of differentiation, the hepatic specification is induced with fibroblast growth factor and bone morphogenetic proteins 2 and 4 (Schwartz et al. 2014; Hannan et al. 2013). To mature the derived cells, culture media can be supplemented with hepatocyte growth factor, oncostatin M, and dexamethasone (Hannan et al. 2013; Si-Tayeb et al. 2010b; Hay et al. 2008). Besides growth factors, small molecules have been proposed for priming hPSCs for hepatic differentiation (Zhu et al. 2009) or they can be applied in each of the differentiation steps (Tasnim et al. 2015). The use of soluble factors in the hepatic differentiation protocols have been a clear focus as they are commercially available and can be easily added into the culture media. However, it is recognized that not only soluble factors but also cell-cell interactions and cell-matrix interactions play an important role in the complex, multistage cell differentiation process (DiPersio et al. 1991). One route to improve the in vitro culture systems is to learn from the ECM cues in vivo, an approach which was successfully demonstrated in this thesis (III, IV).

At the moment, the optimal culture matrix for in vitro hepatic differentiation of the DE cells is poorly understood. A careful analysis of a target tissue can lead to the design of new biomaterials. It has been suggested that a systematic examination of ECM components and their localization in decellularized liver could guide the development of new matrices and engineered culture systems for liver cells (Ananthanarayanan et al. 2011). We hypothesized that ECM produced by human liver progenitors would induce efficient hepatic specification. In hepatic differentiation, the DE cells commit first to hepatic progenitors and thus we aimed to create a progenitor-specific matrix to assist the hepatic lineage differentiation of DE cells. To test the hypothesis, we created an ACM from human liver progenitor HepaRG cells and plated hPSCs-derived DE on the ACM. Indeed, we were able to show that HepaRG-ACM supports attachment of DE cells derived from three different hPSC lines, and also, their hepatic differentiation (III). Cell sheet-derived ACMs are cost-effective but also a laborious approach to produce matrices. In addition, their ECM protein content can vary between batches. Therefore we analyzed the HepaRG-ACM proteins in order to study if the effect of HepaRG-ACM can be achieved with defined ECM proteins.

After a comprehensive screening of the ECM proteins secreted by HepaRG cells, fibronectin, LN-511, and LN-521 were identified (IV). Collagen type IV α2 and α5 chains were also detected in HepaRG-ECM but as they cannot assemble a known collagen protein we did not include collagen type IV in our experiments. The three identified ECM proteins were then used for plating DE cells individually and with all their possible combinations to study if they can replicate the effect of HepaRG-ACM. All the studied matrices supported the attachment of hPSC-derived DE cells and the cells were successfully differentiated towards hepatic cells expressing typical liver markers (IV). Unexpectedly, the differentiation efficacy on defined matrices was even higher compared to ACM (III, IV). This might be due to a loss of ECM proteins during the decellularization process of HepaRG before plating the DE cells.

Laminins have lately aroused interest as cell culture matrices for hepatic differentiation of hPSCs (Vuoristo et al. 2013; Takayama et al. 2013; Takayama et al. 2014; Cameron et al. 2015). In the adult liver, LN-511 and LN-521 are expressed

in periportal area which is suggested to hosts the most immature hepatic-lineage cells (Kikkawa et al. 2008; Turner et al. 2011). Recently, Cameron et al. (2015) used recombinant laminins, LN-521 and LN-521/LN-111 mixture, for plating hPSCs and induced them to hepatocyte-like cells via DE stage. In our study, we seeded the hPSC-derived DE cells to a hepatic progenitor-like environment to provide a stage-specific matrix to guide their differentiation to progenitors. Interestingly, we observed that only LN-511 and LN-521 but not LN-111, support the attachment of DE cells (IV and III, respectively). In addition, the cells failed to attach to surfaces coated with collagen types I and III and Matrigel (III). Matrigel does not contain LN-511/521 which might explain why it inhibited the DE attachment. Matrigel is widely used for culturing many cell types including hPSCs, also in our study. However, during the DE differentiation on Matrigel the cells can remodel the matrix and also, the ECM in the culture well is dissimilar to the original coating substratum. This could explain why the DE cells cultured on Matrigel did not attach to fresh Matrigel coating. However, further studies on ECM expression should be performed to confirm this hypothesis. Collagen type III is expressed in zone 1 like laminins (McClelland et al. 2008), but failed to support the attachment of DE cells (III). Fibronectin, alone and when combined with laminins, supported both the attachment and hepatic differentiation of DE cells (IV). However, as fibronectin is not a liver specific ECM protein and we did not observe improvement in the cell differentiation efficacy with its presence, we did not include fibronectin in our later studies.

The described HepaRG-ACM, LN-511 and LN-521-based matrices showed promising results in terms of efficient hepatic specification (III, IV). The derived progenitors highly expressed transcription factors HNF4A, AFP, and CK-19 (III, IV). In regenerative medicine, there has been increased interest in using hepatic stem/progenitor cell therapies to treat liver damage (Cardinale et al. 2014; Fausto 2004). Recombinant LN-511 and LN-521 would provide an ideal matrix for obtaining hepatic progenitor cells from hPSCs as they are GMP-grade xeno-free reagents. To use the derived hepatic cell in the clinics, our protocol should be further modified so that it would only contain xeno-free reagents in each culture step. For drug and chemical testing, the matrix and cell culture media do not have such limitations but the derived cells should not exhibit batch-to-batch variations. The use of defined matrices can be helpful to reduce variability between the cultures. During the protocol development we optimized the LN-511 and LN-521 coating concentration to improve the differentiation efficacy as the density of ECM proteins is known to regulate the cell fate (Owen and Shoichet 2010). Other optimized parameters to improve hepatic maturation were plating density of DE cells, culture period, growth factor combination for DE induction for each hPSC line, and we also tested Matrigel overlay at the end of the differentiation (III, IV).

Finally, we demonstrated that LN-511 and LN-521 promote the hepatic specification of both hESC and hiPSC-derived DE-cells. In this study we did not compare the differentiation efficacy to any known protocol in our laboratory and that could be done in the future. On the other hand, the control culture would have to be performed in a different way since the DE cells did not survive on any other of the tested matrices than LN-511, LN-521, and fibronectin. The control culture system, for example culturing the hPSCs on Matrigel wihthout the detachment step, would let us compare the end product of the differentiation, derived-hepatocytes, but it would not reveal new information about the specific matrix for DE cells. In our

study, the derived cells increased their ALB secretion over the culture period and exhibited metabolic activity confirming their hepatocyte-like status (IV). However, they were phenotypically closer to fetal hepatocytes instead of mature hepatocytes. Thus, the culture protocol should be further modified to obtain more matured cells. Possible strategies to improve the culture condition could include exposing the cell monolayer to flow in a flat bioreactor, creating a co-culture system, or transfer the cells into matrices composed of zone 3 proteins, such as collagen type I and IV, after reaching the fetal hepatocyte-like status (Ordovás et al. 2013; Gieseck et al. 2014; Takebe et al. 2013).

It is clear that ECM chemistry has an important role in guiding hepatic differentiation in vivo and in vitro. ECM secretion already starts at the embryonic stage, and throughout the development ECM has an essential function in regulating cell functions and maintaining the tissue homeostasis (Rozario and DeSimone 2010; Badylak et al. 2015). In the liver, the ECM chemistry exhibits gradients from zone 1 to zone 3. Learning from these ECM gradients can help us to identify novel biomaterials for hepatic differentiation. Our study serves as an example that it was possible to identify new hepatic lineage stage specific biomaterials by characterizing the ECM proteins of the liver progenitor cells. This approach can be applied to find other new matrices for other specific lineage stages in the liver or in other tissue.

6.2 Multidimensional cell cultures require advanced analysis techniques

To record the data from advanced 3D cell culture systems, special analysis techniques are required (Pampaloni et al. 2007). Imaging of 3D spheroids and tissue samples is especially challenging due to the thickness of a specimen. When the cells are cultured within a biomaterial the matrix can also hamper or even prevent the analysis. For example, for single-cell-based assays such as flow cytometry the cultured 3D spheroids need to be released from the matrix and disintegrated into single cell suspension.

Even though there are numerous biomaterials described for cell culturing, only a few of them are optimal for analysis of 3D spheroids. Some of the commercial hydrogels can be enzymatically degraded enabling more downstream analysis of the cells. We cultured hPSCs and HepG2 cells in HG hydrogel, composing of HA, gelatin and PEGDA, to study the 3D structure of the cells. However, the enzymatic degradation of HG hydrogel with collagenase/hyaluronidase, as recommended by the manufacturer, destroyed the spheroid structure and thus prevented the analysis (II). We hypothesized that spheroid disintegration during collagenase/hyaluronidase treatment might have been caused by a cleavage of collagen and HA which were possibly formed by the cells to stabilize the spheroid structure. However, we have not studied the presence of ECM proteins in the spheroids cultured in HG or NFC hydrogel by staining or PCR. Nonetheless, we have observed an ECM-like network formed around the spheroids cultured in NFC hydrogel (II). The analysis of ECM proteins produced by the spheroids could be performed in the future.

Numerous natural, synthetic, and hybrid biomaterials have been proposed for 3D cell culture. However, hydrogels which allow release of whole 3D spheroids have

rarely been described. D. Wang et al. (2011) introduced a thermoreversible hydrogel from which the intact spheroids can be recovered by incubating the plate at room temperature. The group encapsulated HepG2 cells in thermosensitive poly(N-iso-propylacrylamide) in situ by increasing the temperature to 37°C at which the gelification occurred immediately. The spheroids were released by incubating the culture at 25°C for four hours in total. The described protocol might be applicable for strong cell lines such as HepG2 but could not be used for sensitive cells such as stem cells without decreasing their viability or causing unwanted differentiation. The most prominent feature of NFC hydrogel is that viable intact spheroids can be released from the culture matrix. Plant-derived NFC is composed of fibers that are formed from aligned β-D-glucopyranose polysaccharide chains (Bhattacharya et al. 2012; Pääkkö et al. 2007). Our group showed earlier that NFC hydrogel can be degraded with cellulase enzyme without interfering with the viability, structure, or pluripotency of 3D hPSC spheroids (Lou et al. 2014).

Stabilizing architecture of 3D spheroid for detailed morphological examination has turned out to be challenging. Inspired by single cell silicification demonstrated by Kaehr et al. (2012) we tested if the 3D structure of spheroids can also be preserved to more closely study the morphology of 3D organized cells. Indeed, we showed that SBR is a powerful technique to maintain the intact spheroid architecture, fine cellular structures, and also cellular antigens (II). We combined SBR with SEM imaging for detailed morphological examination of 2D and 3D cultured hPSCs and HepG2 cells. In addition, we showed that all organic cellular material can be removed by high temperature calcination to create identical silica replica of a spheroid. SBR could possibly be combined with other analysis techniques as well, or production of silica replicas could be used to develop highly detailed biomimetic materials as also suggested by Townson et al. (2014).

Releasing 3D spheroids from the culture matrix is beneficial for confocal microscope imaging. We demonstrated that the spheroids recovered from the NFC hydrogel can be imaged as whole-mount samples or sectioned prior to histological analysis (I, II). Optionally, the spheroids, fixed or living, can be stained and imaged inside the NFC hydrogel (I). However, the resolution of the acquired images is lower when the spheroids are inside the NFC matrix which is most likely due to light scattering from the fibers. In addition, penetration of the antibodies might be decreased in the hydrogel. Lastly, the thickness of a whole-mount specimen itself causes challenges in their analysis and is commonly the limiting factor in imaging (Pampaloni et al. 2007). Significant advances in 3D imaging have been obtained with light sheet fluorescence microscopy (LSFM) that is applicable for thick samples, both fixed and living (Pampaloni et al. 2015). In the future, LSFM imagining could be used to analyze intact 3D hPSCs generated in NFC hydrogel. The diameter of hPSC spheroids can exceed several hundred micrometers and with confocal microscope imaging the inner cell mass of whole mount spheroids cannot be detected well. Our group has overcome this challenge earlier by sectioning the spheroids, however LSFM would enable analysis of the whole spheroid.

7 Conclusions

Mimicking liver environment in a dish can be approached from various angles. The native liver can best be resembled by using whole liver ACMs, liver chemistry by ECM proteins, liver structure with fibrillar materials or scaffolds, and liver stiffness, for example, with hydrogels. Indeed, complexity of the liver cannot be captured with standard, simple 2D cell culture techniques. The matrices studied in this thesis, the HepaRG-ACM, LN-511, and LN-521, liver ECM component-based biomaterials, NFC hydrogel, an artificial liver-mimicking biomaterial, and HG hydrogel, hybrid biomaterial, are promising materials to differentiate hepatic cells in vitro (I, III, IV). In addition, this thesis demonstrates a new workflow to find new biomaterials and to develop defined matrices (III, IV). For morphological examination of complex 3D constructions, this thesis suggests a straightforward protocol that uses NFC hydrogel as culture matrix and SBR to preserve intracellular and extracellular structures of the spheroids (II). In summary, this thesis describes new bioinspired matrices for hepatic differentiation in vitro, a protocol to analyze complex spheroid morphology, and that the described three-step approach can guide the identification of new ECM protein-based cell culture matrices.

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