Three-dimensional electrospun nanofibrous scaffolds to fabricate in vitro model for liver diseases

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**THREE-DIMENSIONAL ELECTROSPUN NANOFIBROUS
SCAFFOLDS TO FABRICATE *IN VITRO* MODEL FOR LIVER
DISEASES**

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**INTERDISCIPLINARY GRADUATE SCHOOL
NTU-NORTHWESTERN INSTITUTE FOR NANOMEDICINE (NNIN)**

2020

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SCAFFOLDS TO FABRICATE *IN VITRO* MODEL FOR LIVER
DISEASES**

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Interdisciplinary Graduate School
NTU-Northwestern Institute for Nanomedicine

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Assoc. Prof. Tan Lay Poh

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the second author with equal contribution.

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The contributions of the co-authors are as follows:

- Assoc. Prof. L.P. Tan and Prof. J.A. Wertheim provided the initial project direction and edited the manuscript drafts.
- I co-designed the study with Dr. J.H. Brown and contributed equally to the laboratory work at the School of Materials Science and Engineering NTU Singapore and Northwestern University Feinberg School of Medicine, Chicago. I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me, Dr. Jessica Brown and Dr. D. Ivancic in the Facility for Analysis, Characterization, Testing and Simulation.
- Dr. J.H. Brown prepared the initial manuscript drafts. The manuscript was revised by me, Dr. M.D. DiVito, Prof. J.A. Wertheim and Prof. L.P. Tan.

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Abstract

Liver extracellular matrix, which has random fibrous network structure, can be closely mimicked by the electrospun fibrous scaffolds. Thus, electrospinning is a promising technology to develop scaffolds for liver tissue engineering. However, the closely packed fibrous mat obtained by the conventional electrospinning process restricts cellular infiltration, thus act like a two-dimensional cell culture model which is different from the *in vivo* three-dimensional microenvironment. In this study, an interdisciplinary approach was applied to construct a three-dimensional liver tissue mimicking scaffold by using the knowledge of material science and molecular biology. A newly optimized wet electrospinning process was applied to prepare three-dimensional scaffolds with random fibrous network structure. The three-dimensional random electrospun fibers mimic the physical microenvironment, whereas the extracellular matrix proteins grafted on the fiber surface by chemisorption process mimics the chemical microenvironment for the human liver cells. Collagen-I and fibronectin, which are two essential liver extracellular matrix proteins were used to modify the fiber surface. The initial findings suggested that collagen-I modified 3D wet electrospun scaffolds were superior compared to both fibronectin modified scaffold and gold standard sandwich culture in maintaining functions of primary human hepatocytes *in vitro*. Thus, collagen-I modified 3D electrospun scaffold was used for co-culture of primary human hepatocytes with human hepatic stellate cells to establish the optimum ratio of these two liver cells in the *in vitro* culture. The ratio was optimized by studying the long-term functional maintenance of primary human hepatocytes. The distinct role of collagen-I and fibronectin for *in vitro* maintenance of primary human hepatocytes were analyzed and these findings leads to further exploration of the combined effect of these two-essential ECM proteins for the culture of human liver cells *in vitro*. A series of different ratios of collagen-I to fibronectin were used to chemisorb the electrospun scaffolds. The protein amounts were optimized based on total protein absorbed and the homogeneous distribution of both the proteins on the scaffold surface. Improved protein distribution on the fiber surface was found to enhance functional maintenance of human hepatocytes

on 3D electrospun fibrous scaffolds. The findings suggested that cellular microenvironment controls translational and transcriptional properties of the liver cells. The optimized 3D electrospun fibrous scaffolds with optimum ratio of human primary hepatocytes and human hepatic stellate cells were used to fabricate an alcoholic liver disease model. The proposed model was analyzed at different dosage of alcohol under different scaffold conditions. The effect of alcohol was found to be more prominent on the cells cultured on the electrospun fibrous scaffolds modified with both collagen-I and fibronectin when compared to the single protein modified scaffolds, unmodified scaffolds and the gold standard sandwich culture. This ECM modified electrospun fibrous scaffolds with human liver cells can be used as an engineered liver tissue for studying the effect of different toxins, fibrosis causing elements or dose dependent study of antifibrotic drugs.

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Abbreviations

2D	Two-Dimensional
3D	Three-Dimensional
ALB	Albumin
ALD	Alcoholic Liver Diseases
ALT	Alanine Aminotransferase
BCA	Bicinchoninic acid
BLD	Bile-Duct Ligation
BSA	Bovine Serum Albumin
CCK-8	Cell Counting Kit-8
CHCl ₃	Chloroform
CO ₂	Carbon Dioxide
COL1A1	Collagen-1A1
CYP	Cytochrome P450
DAPI	4',6-diamidino-2-phenylindole
DEN	Diethylnitrosamine
DI	Deionized
DMEM	Dulbecco's Modified Eagle's Medium
DMF	Dimethylformamide
DMN	Dimethylnitrosamine
DNA	Deoxyribo nucleic Acid
DPAC	Diethylpyrocarbonate
ECM	Extracellular Matrix
EDC	N-ethyl-N'-(3-dimethyl aminopropyl) carbodiimide
EDTA	trypsin-ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
ELISA	Enzyme-linked Immune Sorbent Assay
EthD-1	Ethidium Holodimer-1
ETOH	Ethanol

FBS	Fetal Bovine Serum Albumin
FI	Fluorescence Intensity
FTIR	Fourier Transform Infrared Spectroscopy
H&E	Hematoxylin and Eosin
HCC	Hepatocellular Carcinoma
HCM	Hepatocyte Culture Medium
HCV	Hepatitis C Virus
HF Diet	High-Fat Diet
HGF	Hepatocyte Growth Factor
HGF	Hepatocyte Growth Factor
HNF4A	Hepatocyte Nuclear Factor 4 Alpha
HNF4A	Hepatocyte Nuclear Factor 4 Alpha
HSCs	Hepatic Stellate Cells
ICC	Inverted Colloidal Crystal
ICC	Inverted Colloidal Crystal
KBr	Potassium Bromide
LPS	Lipopolysaccharides
MCD	Methionine-Deficient and Choline-Deficient
Mdr2	Multidrug Resistance-Associated Protein 2
MES	2-(N-morpholino)ethanesulfonic Acid
MMPs	Matrix Metalloproteinase
mRNA	Messenger Ribonucleic Acid
NAFLD	Non-Alcoholic Fatty Liver Diseases
NaOH	Sodium hydroxide
NASH	Non-Alcoholic Steatohepatitis
NHS	N-hydroxy succinimide
PBS	Phosphate Buffer Saline
PBST	Phosphate Buffered Saline with 0.1% Tween-20
PCK	Phosphoenolpyruvate Carboxy Kinase
PCK	Phosphoenolpyruvate Carboxy Kinase
PDGF	Platelet-Derived Growth Factor

PDGFR α	Platelet-Derived Growth Factor Receptor α
PEDOT	Poly(3, 4-ethylene dioxythiophene)
PEG	Polyethyleneglycol
PEG	Polyethyleneglycol
PFA	Paraformaldehyde
PHHs	Primary Human Hepatocytes
PLA	Poly-L-Lactic Acid
PLGA	Poly(lactic-co-glycolic)acid
PP	Polypropylene
PVDF	Polyvinylidene Fluoride
qPCR	Quantitative Polymerase Chain Reaction
RGD	Arg-Gly-Asp
ROS	Reactive Oxygen Species
RT	Room Temperature
RT	Room Temperature
RT	Room Temperature
SD	Standard Deviation
SEM	Scanning Electron Microscopy
TCP	Tissue Culture Plate
TGF β 1	Transforming Growth Factor Beta-1
TIMPs	Tissue Inhibitor of Metalloproteinases
TLR4	Toll-like receptor 4
TNF α	Tumour Necrosis Factor- α
T β R-I	Transforming Growth Factor β Type-I Receptor
T β R-II	Transforming Growth Factor β Type-II Receptor

Chapter 1

Introduction

This chapter begins with the background of in vitro liver disease models and its importance in liver tissue engineering followed by the hypothesis of this research (Section 1.1. and 1.2.). The scopes of wet electrospun random fibrous scaffolds in liver tissue engineering is discussed further in research objectives and scopes (Section 1.3.). The significance and novelty of the research also stated (Section 1.4.) in this chapter. Finally, the dissertation overview is outlined to indicate the contents of the subsequent chapters (Section 1.5.).

1.1 Background

In vitro liver disease models comprising of liver cells and supporting scaffolds are becoming the area of significant interest for improving the understanding of disease mechanisms and finding the probable cures [1]. The cell-specific functions of the liver cells depend on the microenvironment provided by the artificial scaffolding system. Thus, finding a model scaffold to support the viable and functional liver parenchymal and non-parenchymal cells is crucial for the construction of *in vitro* liver disease models. Resemblance of three-dimensional (3D) electrospun random fibrous scaffold with the liver extracellular matrix (ECM) is the principal reason for growing interest in the former for application as scaffold in liver tissue engineering [2]. Numerous synthetic and natural polymers has been used so far to fabricate electrospun fibers which further supported the cell adherence at the initially, cell proliferation at the later timepoints, and cellular functions at the translational and transcriptional level [3, 4]. However, the functional behaviors of the liver cells at the translational and transcriptional level under disease conditions on these 3D electrospun fibrous scaffolds at the functional level are still to be explored.

Liver fibrosis known as a wound-healing activity of the injured liver cells, which eventually results in scar tissue formation due to the over deposition of the extracellular matrix [5]. Hepatic stellate cells (HSCs), a non-parenchymal cell of liver trans-differentiating to proliferative, contractile myofibroblast on liver injury is known as the pre-eminent collagen-producing cells present in the liver. On prolonged injury, fibrosis gradually progresses to hepatocellular carcinoma (HCC) which is the fifth most reported cancer type in the industrialized countries [6]. Significant efforts have been taken to find out the molecular mechanisms responsible for disease progression and the therapeutic strategies to disrupt the progression mechanism using both *in vivo* and *in vitro* models [7]. *In vivo* models based on rodents were limited due to their inability to develop many human hepatic diseases e.g. hepatitis C virus infection and oversensitivity towards many fibrosis-inducing parameters e.g. alcohol, bile duct ligation, etc. [8]. To overcome these problems a well representative three-dimensional

in vitro model with human cells are becoming more appropriate to study liver fibrosis. The three-dimensional (3D) *in vitro* models using hepatic stellate cells to investigate liver fibrosis, so far have been focusing on the molecular mechanisms responsible for the fibrosis progression. However, spontaneous activation of the HSCs *in vitro* triggers differential fibrotic gene expression profiles which is different from the *in vivo* counterpart process [9, 10]. The spontaneous activation of HSCs specifically triggered by stiffness of culture plate and two-dimensional (2D) microenvironment which is significantly different from the *in vivo* 3D microenvironment of liver cells. Thus, it may not be able to reflect the *in vivo* pathophysiological mechanisms manifested on liver fibrogenesis. Taken together, cell microenvironment plays a crucial role to maintain functional characteristics of liver cells in both normal and disease conditions for *in vitro* studies.

1.2 Hypothesis

3D electrospun random fibers modified with essential ECM proteins, that closely replicates the native liver extracellular matrix and supports long-term viability of functional liver cells could be used as a disease model to study liver diseases *in vitro*.

1.3 Research Aims and Objectives

The general aim of this research is to fabricate an *in vitro* three-dimensional disease model by mimicking the native microenvironment of the liver cells. Electrospun nanofibers will represent the physical fibrous nature of the native liver ECM whereas the chemically bonded biologically active molecules, present on the fiber surface will promote cell adhesion and liver-specific functional activity. Alcohol treatment was used to study the initiation of fibrosis in HSCs in the presence of primary human hepatocytes (PHHs). Thus, to achieve these goals, three prime aims were identified and discussed as follows:

- 1) **Aim 1:** Fabrication and characterization of 3D electrospun fibers followed by surface modification with biologically active molecules.

Objectives:

- Fabrication of three-dimensional scaffolds by wet electrospinning process with the required diameter and pore size distribution.
- Surface modification of electrospun nanofibers with different concentrations of the Collagen-I and fibronectin by chemisorption process.
- Characterization of 3D electrospun scaffolds, before and after the chemisorption with biologically active molecules.

- 2) **Aim 2:** Investigation of fiber surface modification and co-existence of collagen-I and fibronectin on viability and functionality of human hepatocytes.

Objectives:

- Study on the effect of collagen-I or fibronectin modification on 3D wet electrospun fibrous scaffolds for maintaining of viability and functionality of human primary hepatocytes.
- Optimization of collagen-I and fibronectin ratio for surface modification of electrospun nanofibrous scaffolds with both essential ECM proteins.
- Inspection on the effect of co-existence of both collagen-I and fibronectin on translational and transcriptional properties of human hepatocytes.

- 3) **Aim 3:** Evaluation of the effect of alcohol treatment on the proposed scaffold-cell culture model to analyse alcoholic liver fibrosis *in vitro*.

Objectives:

- Optimization of primary human hepatocytes with hepatic stellate cells co-culture on the liver-specific functional properties of human primary hepatocytes.
- Study on the effect of alcohol treatment on the primary human hepatocytes and human hepatic stellate cells co-culture on protein modified 3D electrospun fibrous scaffolds.

1.4 Significance and Novelty

Hepatocytes based treatments for liver diseases have been highly restricted by the short life span of human primary hepatocytes and losing hepatocyte-specific functions at the early time points of *in vitro* cultures. This dissertation demonstrates that electrospun random fibers modified with native extracellular components can create a closely mimicking *in vivo* microenvironment to support the functionality and viability of primary human hepatocytes in long term culture. This technology also provides a tunable microenvironment with one or more native ECM proteins for *in vitro* maintenance of human liver cells. The amount of each ECM protein plays a significant role in the homogeneous distribution of the proteins on an uneven surface like fibers. It is also found to affect the total protein retention after surface modification of electrospun fibers. Collagen-I and fibronectin showed differential effect in the functional regulation of human primary hepatocytes, whereas the presence of both the proteins on the same platform found to be beneficial over the single protein modified culture groups. Additionally, the effect of fibrosis-inducing substrates (i.e. alcohol) on PHHs/HSCs co-culture systems has been investigated. The response of co-culture on different scaffolds has been analyzed. The results were compared with the gold standard for *in vitro* culture of human liver cells. Thus, the novelty of the reported study is the fabrication of a scaffold system to preserve the long-term functional and viable human liver cells which in turn can be used to study the effect of different fibrosis causing components *in vitro*.

1.5 Dissertation overview

This thesis incorporates of the following eight chapters:

Chapter 1 includes the backgrounds of this study followed by the hypothesis. A brief introduction to the research objectives and scopes is discussed along with the significance and novelty of this research. Finally, an outline of the thesis is presented.

Chapter 2 contains detailed literature review relevant to the tissue engineering approaches in studying liver fibrosis. The functions of liver cells in liver fibrosis is discussed for *in vivo* and *in vitro* models. The role of alcohol in causing liver fibrosis is highlighted with both *in vivo* and *in vitro* models on alcoholic liver disease.

Chapter 3 presented the overall experimental section including materials, methods, and techniques involved in this dissertation.

Chapter 4 demonstrates the effect collagen-I and fibronectin modified nanofibrous scaffolds on viability and functionality of primary human hepatocytes.

Chapter 5 indicates the advantageous effect of the co-culture of human hepatic stellate cells (HSCs) and primary human hepatocytes (PHHs) on the functional maintenance of the later in *in vitro* culture model.

Chapter 6 presented the synergistic effect of collagen-I and fibronectin, which are two important constituents of liver extracellular matrix, in the maintenance of long-term cultures of human primary hepatocytes. Optimization of the ratio of collagen-I and fibronectin for the modification of electrospun nanofibers has been established in this chapter.

Chapter 7 demonstrate the effect of alcohol on human hepatic stellate cells (HSCs) and primary human hepatocytes (PHHs) in co-culture on protein modified electrospun scaffolds. A dose-dependent study of alcoholic fibrosis was presented and discussed.

Chapter 8 briefly discussed and summarizes the results and concludes the outcomes of this study. It also includes future scopes and prospective.

Chapter 2

Literature Review

This chapter presents a detailed discussion of literature to present an overview of liver fibrosis and the approaches to study the same. It starts with a brief discussion of liver anatomy followed by an introduction to liver fibrosis. It enlightens the different factors affecting the trans-differentiation and perpetuation of Hepatic stellate cells (HSCs). Advancement in understanding of liver fibrosis and investigation of effective clinical approaches to cure the fibrosis is discussed. On this context, a detailed discussion of in vivo and in vitro models of liver fibrosis is reviewed in this section. Scaffold based in vitro models are introduced along with the importance of fiber-based scaffolding systems for in vitro experimentations. Finally, different cell lines evaluated for in vitro fabrication of liver disease model is listed and explained briefly.

2.1 Architecture of the liver

Liver is the largest internal gland and organ present in the human body. It is present in the upper-right portion inside abdominal cavity and under the diaphragm to the right side of the stomach. It is responsible for 2-3% (~1.4-1.6 kg) of the total body weight [11] plays an important role in protein synthesis [12], detoxification [13], digestion and various other types of metabolic activities. The soft liver tissues are reinforced by the peritoneum of the abdominal cavity. There are four lobes present in the liver. Among them left and right lobes are largest and separated from each other by the falciform ligament (**Figure 2.1**) [14].

Figure 2.1: Front and bottom view of the liver anatomy

Liver lobes are made of different types of parenchymal and non-parenchymal cells dispersed in a multi-component extracellular matrix (ECM). It plays an crucial role in retaining the dimensional stability of the organ [15]. Hepatocytes are parenchymal cells, consists of 70-85% of the total liver volume, responsible for a massive number of metabolic activities and detoxifying mechanisms taking place in liver [16]. It has been reported that hepatocytes have shown an extensive regenerative capability and able to regenerate even after a 70% loss of the liver mass [17]. Kupffer cells, sinusoidal

endothelial cells, liver associated lymphocytes and hepatic stellate cells are the non-parenchymal cell of the liver that constitute 6.5% of the total liver volume and 40% of the total cells present in the liver. These cells have important roles in liver growth and regeneration, not only by controlling their proliferation but also by influencing the growth of the parenchymal cells with soluble mediators and direct cell-cell contact [18]. Liver ECM which consists of macromolecular proteins, provide scaffolding to the liver cells. Broadly, the ECM components can be divided into two types. They are collagenous, i.e. the collagen-based proteins and non-collagenous i.e. the proteins that have different amino acid sequence other than collagen. Collagen I, III and V are ECM building proteins, present extensively in liver. Fibrillar collagens have uninterrupted triple helix structure that provides mechanical strength to ECM. The non-fibrillar collagens, which are also known as network-forming collagen (e.g. collagens IV, VI, and VIII) have irregularities in their triple-helical chains. This results in structural flexibility, enable them to make linear, axial, lateral associations. This results in the formation of network structure instead of fibers [19].

Among the non-collagenous proteins, the most abundant and widely distributed ECM glycoprotein is fibronectin, which has multiple domains to crosstalk with cells as well as proteins present in the extracellular matrix. It has a collagen-binding domain, a heparin-binding N-terminus and an Arg-Gly-Asp (RGD) motif-containing cell-binding domain with repeated sequences known as type 3 repeats. Other than fibronectin, laminins, elastin, fibrillin, proteoglycans, Tenascin-C, hyaluronic acid, decorin etc. are also a part of non-collagenous proteins [20, 21]. The amount of different ECM components in a decellularized liver is presented in **Figure 2.2** [21].

Figure 2.2: Distribution of ECM components in the healthy liver

2.2 Liver Fibrosis

Liver fibrosis is a wound healing response that takes place due to repeated or prolonged injury of the liver [5]. Common causes of liver fibrosis reported so far are alcohol abuse, virus attacks such as hepatitis B, hepatitis C virus, diabetes, metabolic syndrome, non-alcoholic steatohepatitis (NASH) and chemical toxicity due to drug overdose [22]. On persistent or chronic injury, the fibrosis proceeds towards cirrhosis and results in hepatocellular carcinoma (HCC). It is the fifth most common cause of death in the industrialized countries [23]. Briefly, the liver disease progresses through inflammation, fibrosis and finally cirrhosis stages (**Figure 2.3**). Fibrosis is a reversible condition and it is possible to restore the functions of the liver by treatment at the early stages of cirrhosis. On mild injury, neighboring adult hepatocytes regenerate itself to substitute

the apoptotic and necrotic cells. However, on chronic or persistent insult the rate of regeneration of the hepatocytes become slower than the rate of scar tissue formation. This eventually results in cirrhosis followed by liver failure. The only treatment available for end-stage liver cirrhosis is liver transplantation. However, the scarcity of donor organs is the prime challenge. Thus, it becomes very important to find out new technologies to treat liver fibrosis and to diagnose the liver diseases at the early stages of the injury [24].

Figure 2.3: Activation of hepatic stellate cells by soluble mediators from injured hepatocytes

Activation of hepatic stellate cells (HSCs) has been identified as the central event to initiate hepatic fibrosis. It is a non-parenchymal cell and occupies 5-8% of the total liver cell population [25]. In a healthy liver, HSCs inhabit the space of disse, stores vitamin A in the form of retinoid droplets. Activation of HSCs includes two major phases. They are initiation and perpetuation and finally resolution if the injury attenuates by application of the suitable antifibrotic agent. HSCs in the quiescent state are less proliferative. Activated HSCs has shown excessive proliferation and secretion of extracellular matrix proteins which is mainly comprised of fibrous collagen-I. It causes scar tissue formation and liver hardening [26]. The apoptotic bodies and signals obtained from injured hepatocytes result in upregulation of collagen expression. It

enhanced the production of reactive oxygen species (ROS) by upregulation of cytochrome P450 2E1 (CYP2E1). ROS increases the proliferation and collagen synthesis by HSCs [27]. Among different fibrogenic cytokines released by the apoptotic hepatocytes, one of the most potent fibrogenic cytokines is transforming growth factor beta-1 (TGF β 1). It remains stored in the hepatocytes as well as in the extracellular matrix of the liver as a large latent complex. Hepatocytes, liver macrophages (kupffer cells) and ECM matrix proteins release an abundant amount of TGF β on liver injury. This is considered as the primary signal to HSCs activation [28, 29].

The release of TGF β by the hepatocytes on liver injury is known as the pre-inflammatory phase of the HSCs activation. TGF β down-regulates the expressions of matrix metalloproteinase (MMPs) which are responsible for the degradation of the ECM proteins. Expression of tissue inhibitor of metalloproteinase's (TIMPs) in HSCs as well as in myofibroblasts increased on liver fibrosis [30]. Platelet-derived growth factor (PDGF) act synergistically with TGF β to enhance the collagen I synthesis in liver fibrosis. TGF β also induces the apoptosis of hepatocytes and act as a negative regulator for the proliferation of hepatocytes [31]. Activated HSCs itself produces TGF β 1 along with different types of profibrogenic cytokines such as PDGF, Fibroblast growth factor (FGF), etc. This, in turn, promotes the advancement of the hepatic fibrosis [32].

2.3 Factors affecting the activation and perpetuation of Hepatic stellate cells (HSCs)

Liver fibrosis is a complex biological process. Several internal and external factors influence it. Some of these factors were influential during the perpetuation of the disease and are discussed below.

2.3.1 Reactive oxygen species (ROS)

Hepatocytes generate ROS because of oxygen-based metabolism. ROS act as a secondary messenger in the cell-signaling cascade. It upregulates the effect of

inflammatory cytokines such as TGF β , PDGF, EGF, TNF α , etc. [33]. ROS plays an important role in HSCs activation by a pro-oxidant agent or accumulation of oxidative stress. In the injured liver, macrophages and infiltrating monocytes generate ROS. It shows a significant effect, on the modulation of collagen production by HSCs [34, 35].

2.3.2. Lipopolysaccharides (LPS)

LPS is a cell wall component of the Gram-negative bacteria [36]. Toll-like receptor 4 (TLR4) is a pattern recognition receptor for LPS. LPS derived from Gut microflora (a microorganism present in the digestive tracts of the human) enters the portal circulation and directly activates HSCs through TLR4. It forces HSC's to generate inflammatory cytokines. It also induces the fibronectin production of HSCs after activation [37, 38].

2.3.3 Apoptotic bodies

Liver injury results in the death of hepatocytes, which then forms apoptotic bodies. Liver macrophages engulf these apoptotic bodies. These liver macrophages generate TGF- β , which is one of the prime fibrogenic cytokines responsible for HSCs activation. HSCs themselves participate in the phagocytosis of apoptotic bodies by engulfing them. It triggers the propagation of myofibroblast in the liver by upregulation of TGF- β 1 and procollagen-1 expression [39, 40].

2.3.4 Transforming growth factor β (TGF β)

TGF β is a multifunctional cytokine. It is the principal stimulator for activation of HSCs on liver diseases. The TGF β released by the hepatocytes, Kupffer cell, inflammatory cells on liver injury are latent and a non-covalent complex of mature TGF β with latency-associated peptide (**Figure 2.4**) [28-31]. Mature TGF β released from the latent complexes due to action of ROS [41], MMP-2, MMP-9 [42, 43], thrombospondin-1 [44], plasmin [45] and also due to the change in pH (especially in acidic pH) [46, 47]. Mature TGF β when bound to the transforming growth factor β type-II receptor (T β R-

II) results in trans phosphorylation and heteromerization of the transforming growth factor β type-I receptor (T β R-I). The signal propagates via phosphorylation of the receptor associated SMAD proteins, which are smad2 and smad3. Smad2/3 undergoes oligomerization with a common mediator i.e. Smad4. The Smad oligomer translocate into the nucleus and bind to the DNA or DNA associated binding proteins to upregulate the target gene expressions. TGF β signaling pathway induces activation of HSCs to its trans-differentiation to the myofibroblast. It promotes ECM deposition; down-regulates ECM scavenging MMPs and up-regulates the expression of TIMPs [28, 29, 48].

Figure 2.4: TGF- β complex: activation and deactivation

2.3.5 Platelet-derived growth factor (PDGF)

Platelet-derived growth factor (PDGF) is known as the most potent cytokine responsible for the proliferation of activated HSCs [49]. Quiescent HSCs shows Platelet-derived growth factor receptor α (PDGFR α) which remains unchanged on activated HSCs. The β subunit of this receptor (PDGFR β) remains undetected in the

Quiescent HSCs and expressed on liver injury. It is a prerequisite for the mitogenic effect of PDGF on HSCs, was evident by the fact that PDGF does not affect the HSC proliferation in the initial stages of the culture [50, 51]. TGF β synergized the effect of PDGF on HSC proliferation [31].

2.3.6 Tumor necrosis factor (TNF)- α

Tumor necrosis factor (TNF)- α is produced by different hematopoietic and non-hematopoietic cells e.g. natural killer cells, endothelial cells, T cells, fibroblasts, neutrophils; were found to have elevated expression on liver injury. It has a controversial part in the progression of liver fibrosis [52, 53]. On one side it acts as a negative regulator of collagen $\alpha 1(I)$ mRNA expression in HSCs and known as an important cytokine in liver regeneration, whereas on another side it behaves as a positive regulator to elevate expression of Tissue inhibitor of metalloproteinase (TIMP)-1, an inhibitor of most of the MMPs. Thus, it prevents degradation of the excessive ECM formed during fibrosis, one of the major reasons for scar tissue formation [54]. Like TGF β , TNF α prevents the apoptosis of the activated and proliferating HSCs that promotes liver fibrosis [29].

2.3.7 Leptin

Leptin is an adipocyte-derived hormone of 16 kDa molecular weight which acts as a regulator for food intake and energy balance of healthy human body [55]. On liver injury, activated HSCs produce leptin, which is not produced by the inactive HSCs, hepatocytes or by liver macrophages [56]. Leptin further promotes the trans-differentiation of HSCs to myofibroblast and proliferation of the latter mediated by activation of the long form of the leptin receptor. It also elevates TGF β and $\alpha 2(I)$ collagen gene expressions in *in vitro* culture. The α -SMA positive cell was not detected in the absence of leptin and vice versa after treating the HSCs with carbon tetrachloride (CCl₄) [57].

2.3.8 Mechanical stress

Physical properties of the substrate influence HSCs phenotype *in vitro*. HSCs adhesion on stiff substrate induces mechanical stress resulting in activation and trans-differentiation. Mechanical tension generated in cells cultured on the stiff surface and restricted by the ECM stimulates cell growth, motility, differentiation, viability, etc. [58, 59]. Portal blood pressure increases on fibrosis and cirrhosis evident from many *in vivo* studies. HSCs present in the hepatic sinusoids experience mechanical tension as the portal pressure increase results in enhanced TGF β secretion, which is the major cytokine to stimulate excessive ECM generation by HSCs on liver injury [60].

2.4 Recent advancement in the study of liver fibrosis

2.4.1 *In vivo* models for liver fibrosis

In vivo models have been extensively used from past few decades to investigate the event cascades in human liver fibrosis due to its ease of use, proximity to the mechanisms involved in human liver and high reproducibility [61]. Chemical-based, diet-based and genetically modified *in vivo* models have been discussed by various groups.

Chemicals such as ethanol, carbon tetrachloride, thioacetamide, dimethylnitrosamine (DMN) and diethylnitrosamine (DEN) have been widely used to generate fibrosis in animal models[7]. Among these chemicals, ethanol treatment is one of the most studied chemical fibrosis models due to immense reports of alcoholic liver diseases in industrialized countries. In rodents, ethanol-induced fibrosis are generated by injecting alcohol directly to the blood stream[62] or by feeding ethanol with water[63-65], although, rodents have a natural aversion towards alcohol consumption except for HAP-2[66] and C57BL/6 [67] mice which were extensively used for alcoholic liver disease models. However, there are no rodent models available to replicate human alcoholic liver fibrosis completely due to species difference and reduced complexity in

the former. Carbon tetrachloride (CCl₄) is the next widely used chemical for replicating liver fibrosis in rodents. It closely mimics the aspects of the toxic damage of human liver in rodents. CCl₄ damage is associated with the formation of trichloromethyl radical that creates an event flow resulting in necrosis of hepatocytes and activation of inflammatory response [68, 69]. This results in the activation of hepatic stellate cells followed by liver fibrosis [70]. Rats and mice are treated with CCl₄ for weeks by oral administration [71], injection [72] and even by inhalation [73]. CCl₄ toxicity results in large lipid droplet formation in both rat and mice liver cells that lead to fatty liver disease with an increase in the relative weight of the liver. Among various models, inhalation of CCl₄ over 6-13 [73] weeks was found to be the best to study cirrhosis such as portal hypertension and ascites formation, although operator training and appropriate equipment to regulate the dosage over time is still to be investigated [74, 75]. CYP P450 enzymes play a significant role in the bioactivation of chemicals to become toxic [76]. Similarly, like CCl₄, thioacetamide degraded by CYP P450 enzymes to Sulphur dioxide is responsible for *in vivo* toxicity. It upregulates the proteins involved in lipid peroxidation and oxidative stress and down-regulates the proteins involved in methionine breakdown, fatty acid β -oxidation [77]. Oxidative damage further results in the activation of HSCs which is the key event in liver fibrosis. Like CCl₄ treated rats, thioacetamide treatments also results in elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) release in blood serum after treatment of 6-8 weeks with a dosage of 100-200 mg/kg of body weight [78]. DMN and DEN are other frequently used chemicals to induce fibrosis in animal models. Abundantly produced ROS during biotransformation of DMN and DEN reacts with proteins [79], lipids [80] and nucleic acids [81] causing generation of centrilobular necrosis as well as cell malfunction. Overall, the chemically treated fibrosis-inducing models are based completely on the higher generation of reactive oxygen species which in turn upregulates fibrotic gene expressions in HSCs results in activation.

Diet-based rodent models are dominated by the strain variations which leads to diet-induced liver fibrosis [82, 83]. Many non-alcoholic fatty liver diseases (NAFLD) and non-alcoholic steatohepatitis (NASH) was developed in rat and mouse models by

following different dietary regulations. Methionine-deficient and choline-deficient (MCD) diet models are frequently addressed by many research groups, however, these mice models are unable to develop some of the human pathological characteristics, such as obesity and pronounced peripheral insulin resistance which are common models to study NASH [84]. MCD diet increases lipotoxicity and steatosis by increasing the generation of triglycerides and this leads to hepatic stress[85]. Kupffer cells are the first to respond to MCD diet by increasing the collagen deposition due to increased production of MMP-13, monocyte recruitment and upregulated production of fibrogenic cytokine TNF- α [86]. 8-weeks of MCD diet leads to steatohepatitis in mice followed by the development to more advanced fibrosis after 16 weeks [87]. To overcome the shortcomings of MCD diet, another diet came into play, i.e. the high-fat (HF) diet which can induce human features such as obesity and peripheral insulin resistance in mice but not in rats. However, in mice it takes nearly 50 weeks to develop steatosis by following HF diet [88]. In rats, a high cholesterol diet was used to develop NASH, although it still lacks the obesity and peripheral insulin resistance development in rats [89]. The choline-deficient L-amino acid defined diet is another diet which is very much similar to MCD diet, but capable in creating obesity and peripheral insulin resistance in both rats and C57BL/6J mice making this model suitable for the study of progression of NAFLD to NASH and to hepatocellular carcinoma (HCC)[90, 91].

Another very commonly used model to study liver fibrosis is bile-duct ligation (BDL) which was first developed in rats and later used in mice [92]. A doubly ligated bile duct bisect between two ligatures results in BDL and causes an increment in biliary pressure which leads to the secretion of fibrogenic cytokines by biliary epithelial cells. This increases profibrotic gene expressions such as TIMP-1, α -SMA, collagen 1 and TGF β 1 [93]. It causes accumulation of T-cells and B-cells in portal tract that generates ROS and injure the liver [94]. Portal fibroblasts, a source of myofibroblasts in BDL produces IL13 causes further activation of HSCs [70]. Although removal of an obstruction in BDL can restore the liver in mice models even after 2 weeks, rupture of the gall bladder or biliary cysts, leakage of bile may cause early mortality of the mice in these models.

Thus, BDL is useful for the short-term study of fibrotic liver under cholestatic injury [70, 95, 96].

The most useful tool to create *in vivo* models so far developed is by using genetically modified rodents. Improved insights in the role of specific proteins or signaling pathways in liver fibrosis were achieved by using genetically modified animal models [97]. However, genetic modifications alone do not result in generation of fibrosis. Further stimulation is required for disease initiation [98]. Mice models deficient in mouse multidrug resistance-associated protein 2 (Mdr2) which is a homolog of human adenosine triphosphate-binding cassette subfamily B member 4 gene, is used extensively to study portal inflammation and proliferation, hepatocyte necrosis, onion-skin-type periductal fibrosis, etc. [99, 100]. Mdr2 deficient mice (at the age of 4-8 weeks) with overexpression of HSC activation markers such as TGF β , α -SMA, COL1A1, PDGFR β develop biliary fibrosis. This mice model can also develop HCC at the age of 4-6 weeks [101, 102]. Alms1Fat^{ausi} mutant mice, another variation of genetically modified rodent model has been used to achieve high serum levels of alanine transaminase, hyperphagic obesity, hypoadiponectinemia, diabetes, pericellular and pericentral fibrosis on HF diet [103]. This variation can develop adipose restriction, promotes lipid flux in liver and decrease in serum adiponectin levels only after 24 weeks of HF diet [104]. Presence of cholesterol further causes the transition of NAFLD to NASH in this diet. Thus, a combination of diet with genotype governing liver injury enables insight into various factors in this model [104, 105].

2.4.2 *In Vitro* three-dimensional matrices to study liver fibrosis

2.4.2.1 Hydrogels

Hydrogels are networks of polymer chains and can absorb a very high amount of water. Mechanical stiffness of the scaffold was found to be one of the factors that have a significant effect on activation of hepatic stellate cells for *in vitro* studies [58]. Hydrogel platforms were principally evaluated for stiffness dependent activation of the hepatic

stellate cells over a definite stiffness range such as 3 kPa (similar to the healthy liver) to 20 kPa (similar to the diseased liver) [59, 106]. Hydrogels derived from natural ECM components e.g. collagen gel, matrigel, maleic anhydride-modified hyaluronic acid was able to maintain the quiescent state of the stellate cells [107]. Friedman et al. has investigated the components of Matrigel and found that they were able to maintain the quiescent phenotype of rat hepatic stellate cells for 7 days. Plastic surface and collagen gel promoted a higher amount of matrix deposition by the HSCs when compared to the Matrigel culture [58]. Perepelyuk et al. reported a visible light-cured system of maleic anhydride-modified hyaluronic acid with dynamic stiffening to study the phenotypical change of rat hepatic stellate cells with increasing matrix stiffness. However, naturally derived hydrogels always face the challenges of high batch-to-batch variation, faster enzymatic degradation and poor mass transfer of nutrients and oxygen [59].

To overcome these disadvantages, scientists have investigated some synthetic hydrogels derived from biocompatible polymers. Liu et al. reported on the use of a multilayer photo patterned polyethyleneglycol (PEG) hydrogel structure containing RGD peptide sequence to culture rat primary hepatocytes with other non-parenchymal cells. The RGD modified hydrogels were superior in maintaining the liver-specific functions such as albumin and urea syntheses of hepatocytes when compared with unmodified PEG hydrogel [108]. Olsen et al. used polyacrylamide hydrogel with different concentration of *bis*-acrylamide to modulate the stiffness of the matrix. It was found that the increased stiffness results in trans-differentiation of the rat hepatic stellate cells. TGF- β did not induce myofibroblastic differentiation on the soft matrix *in vitro* [58]. Generally, very few available data were found to support hydrogel as a matrix for liver cells. Again, enhancement of stiffness with fibrosis progression is a result of hepatic fibrosis whereas the diseases models are principally focusing on the causes of fibrosis and its resolution. Thus, stiffness modulation of the hydrogel may not be considered as a representative model for hepatic fibrosis. Another major limitation with hydrogels platforms, be it synthetic or natural, is that they are not able to mimic the fibrous hierarchical structures of native ECM.

2.4.2.2. Scaffolds other than hydrogel

To overcome the challenges faced with hydrogels, researchers have developed porous scaffolds by combining synthetic and natural materials. Natural materials derived from native ECM act as a cell recognition motif whereas synthetic material gives mechanical strength and dimensional stability to the scaffold. Rad et al. have investigated the use of a blend of hyaluronan, (3, 4-ethylene dioxythiophene) PEDOT, and collagen (I) as dopants in gelatin–chitosan-based scaffold to increase the electrical communication of the GS5 cells (a liver carcinoma cell line). PEDOT, which is a conducting polymer improves cell attachment. These scaffolds showed a significant degradation (50%) from day 1 up to day 14 even in the presence of PEDOT, which is a synthetic polymer [109].

Figure 2.5: SEM images of decellularized rat liver: (a) vessel, (b) a section featuring bile duct-like small vessels (arrows), (c) extracellular matrix within the parenchyma, with hepatocyte-size free spaces. Scale bars: 20 μm (a-c) [109].

Uygun et al. have reported that decellularized liver has a network structure mainly fabricated with fibrous and non-fibrous collagen proteins (**Figure 2.5**) [110]. Eda et al. used a nanofiber membrane prepared by electrospinning of poly(caprolactone) as a scaffold to mimic the network structure of the liver ECM. Rat primary HSCs were found to have a reduced level of α -smooth muscle (α -SMA) and collagen-1A1 (CoL1A1) gene expressions when compared to the HSCs cultured on uncoated polypropylene (PP).

On activation of HSCs, α -SMA and COL1A1 gene expressions were upregulated considerably thus, act as a marker for myofibroblasts. However, the proliferation rate of HSCs on nanofiber membrane was found to be similar to the growth rate of activated HSCs. Thus, the model was unable to support the fully quiescent HSC phenotype. This was probably attributed to the absence of biological cues and its two-dimensional architecture [111]. Mazza et al. investigated the use of decellularized human liver, a natural 3D scaffold to culture liver cells *in vitro*. Decellularization of the human liver was achieved by Perfusion technique. LX-2, a human hepatic stellate cell line cultured in the natural scaffold shows improved viability and proliferation up to day 21 [112]. However, the phenotype of the LX-2 was not further analyzed to confirm maintenance of the stellate cells in its quiescent form. The prime challenge with completely natural decellularized ECM is its batch-to-batch variability, low infiltration of cells and faster enzymatic degradation in the culture conditions.

2.4.3 *In vitro* three-dimensional disease models to study hepatic fibrosis

Extrapolation of *in vitro* fibrosis models to the *in vivo* disease situation needs important cells, cytokines and matrix systems. The designed microenvironment should mimic native tissue architecture. Hepatocytes in 2D monolayer culture were found to lose their liver-specific functions such as specific gene expressions, drug metabolism efficiency within a very short period [113, 114]. The *in vitro* fibrosis models of hepatic stellate cells in monolayer culture on plastic dishes are less representative of the *in vivo* disease progression cascade due to its uncontrolled spontaneous activation [58]. To overcome these challenges researchers investigated many three-dimensional monoculture or co-culture systems to study hepatic fibrosis *in vitro*. Lee et al. proposed a spheroid based microfluidic chip with rat primary hepatocytes and hepatic stellate cells to investigate the role of HSCs in the recovery of alcoholic liver diseases (ALD). It provides a critical value for alcohol dosage to induce reversible (60 μ l/ml) and irreversible (80 μ l/ml) ALD [115]. However, the correlation of cell number with the alcohol dosage was not established in this model. Again, rats have a total aversion of alcohol which is not representative of human cells. The high alcohol level in the blood was not achieved due

to the faster metabolism of alcohol. Thus, extrapolation of the proposed model to the behavior of the human cells may not be acceptable. Bell et al. investigated a 3D spheroid culture of primary human hepatocytes on a microfluidic chip. It maintains the hepatocyte-specific functions for over 5 weeks. In the proteome level, a relatively smaller number of hepatic metabolisms was expressed differentially when compared to a 2D monolayer culture system. 2D culture system showed a differential expression for most of the proteins only after 24 hrs. of the culture period. The size of the spheroid decreased gradually due to the loss of primary hepatocytes. In the co-culture system, the stellate cells and Kupffer cells present inside the hepatic spheroid, restricts the investigation of the role of HSCs on liver injury and fibrosis. However, this model explains the importance of a 3D culture system for human liver cells over 2D monolayer culture [116]. Chang et al. investigated migration of the LX-2 cells in fibrotic microenvironment by using a Boyden chamber. Polyvinylpyrrolidone-free polycarbonate membranes with 8 μm pores were used to separate the fibrotic media contains fibrotic cytokines such as PDGF, TGF- β 1, EGF, VEGF, bFGF (Bottom of the well) and non-fibrotic (Top of the well) cell culture media. The upper side of the membrane mimics the native basement membrane of the liver and had a coating of collagen-IV. Activated HSCs migrate from the top layer to the bottom layer, which explains the migratory properties of HSCs during fibrosis. However, the model was only investigated for a period of 4 hrs. which is not enough for fibrosis progression. Moreover it was unable to maintain the quiescent state of the stellate cells in the culture conditions [117]. Liete et al. generated a scaffold-free hepatic spheroid model termed as a hepatic organoid model with 2:1 human hepatic stellate cell and HepaRG™ cells to analyze drug-induced hepatic injury. The spheroid culture with $190 \pm 10 \mu\text{m}$ size was maintained over 21 days. The researchers were able to show the hepatotoxicity induced activation of hepatic stellate cells in this model [118]. However, the mechanism of HSC activation and the fibrogenic factors involved in this event remain elusive.

2.4.4 Type of Cell lines available to study liver fibrosis

The liver consists of different types of parenchymal and non-parenchymal cells. Liver cells have important roles to play in the normal liver as well as in the fibrotic liver. An *in vitro* disease model should consist of disease-inducing cells or combinations of cells. Hepatic stellate cells, a non-parenchymal cell of the liver plays crucial role in fibrosis development[37]. On activation, HSCs trans-differentiated into myofibroblasts which is α -smooth muscle positive (α -SMA). Activated HSCs produce a large number of extracellular matrix proteins which results in scar tissue formation. Thus, the disease models studied by the researchers on liver fibrosis mainly consists of primary HSCs or the cell lines derived from the same listed in **Table 2.1** [5].

Table 2.1: Primary and immortalized cells lines available to study liver fibrosis *in vitro*

Sl No.	Cell line	Species	Source	Method	Retinoid metabolism	References
1.	LI90	Human	Hepatic mesenchymal tumour	Spontaneous Immortalization	Inducible	[118, 119]
2.	GREF-X	Human	Primary MFB of normal liver	Modification with the large T antigen of polyomavirus	Not reported	[120]
3.	hTERT-HSC	Human	Primary HSC of normal liver	transfer of Retroviral by human telomerase reverse transcriptase	Not reported	[121]
4.	TWNT-1	Human	LI90-cells	transfer of Retroviral by human telomerase reverse transcriptase	Not reported	[122]
5.	LX1	Human	Primary HSC of normal liver	SV40 large T antigen is used for the modification	Inducible	[123]
6.	LX-2	Human	Primary HSC of normal liver	Used Low serum selection pressure in the process as used for LX-1 cells	Inducible	[124]
7.	GRX	Mouse	MFB of fibrotic lesions induced by	Spontaneous immortalization	Inducible	[125-127]

			schistosomiasis			
8.	A640-IS	Mouse	Primary HSC from normal liver	SV40 large T antigen is used for the modification	Not reported	[128]
9.	CFSC	Rat	Primary HSC of CCl ₄ treated cirrhotic liver	Spontaneous Immortalization	Not reported	[129, 130]
10.	NFSC	Rat	Primary HSC of normal liver	Spontaneous immortalization	Not reported	[131]
11.	HSC-T6	Rat	Primary HSC of normal liver	Transformation with SV40 large T antigen	Inducible	[132, 133]
12.	PAV-1	Rat	Primary HSC of normal liver	Spontaneous immortalization	Not reported	[134]
13.	MG-2	Rat	Primary HSC of normal liver	Spontaneous immortalization	Not reported	[135, 136]

Many *in vitro* models have been reported so far to understand the event cascades on liver injury [137]. However, these studies lacked the ability to closely mimic the native liver microenvironment for supporting long-term maintenance of functional liver cells. In *in vitro* studies to maintain functions of liver cells, an *in vivo* mimicking environment is the preliminary requirement [138, 139]. This work emphasized on the preparation of a liver extracellular matrix mimicking scaffold prepared by electrospinning process; surface modified with active biological protein molecules. Liver ECM is mostly rich in collagen-I, responsible for many cell-matrix interaction processes and mechanical regulations [110, 140]. Collagen-I modification on PLGA scaffolds was found to increase urea and albumin synthesis in *in vitro* culture when compared to unmodified PLGA or 2D cultures [141]. However, collagen-I was found to be less specific for attachment of rat primary hepatocytes compared to other types of collagen-coated surfaces such as Collagen-II, III, IV or V [142]. Another important protein of liver ECM is fibronectin which promotes cell-material interaction and maintenance of liver-specific cellular functions in *in vitro* cultures [142, 143]. Elevated levels of albumin and alpha-fetoprotein secretion was achieved by rat fetal hepatocytes and these cells acquired elongated cord-like phenotype when cultured on fibronectin-coated substrate [144]. Activated hepatic stellate cells also showed an elevated level of collagen-I and fibronectin secretions under fibrotic conditions [107]. Thus, collagen-I and fibronectin

modified 3D fibrous scaffolds could be an efficient tool to study disease behaviors of liver cells *in vitro*.

Human hepatic stellate cells cultured on ECM modified 3D PLGA scaffolds are expected to maintain its quiescent phenotype and other differential gene expressions when compared to a 2D culture system or an ECM associated protein unmodified 3D culture system. This cell-ECM assembly could be used as a tool to study the effect of various fibrogenic cytokines and chemicals on liver fibrosis. It will also provide the platform to investigate dose-dependent recovery or apoptosis of the HSCs on the application of an antifibrotic drug.

Chapter 3

Experimental Methodology

This chapter comprises all the main experimental methods and technologies used in the present work. It comprises four sections. The first section starts with the preparation and surface modification of electrospun scaffolds followed by the second section which summarizes the characterization techniques used to optimize the surface modification technique. In the third section, mono-culture studies are included along with co-culture studies of liver parenchymal and non-parenchymal cells. Finally, the fourth section demonstrates the experimental protocol used to analyze and characterize the effect of alcohol treatment on liver cells, statistical significance study among different groups.

3.1 Preparation and surface modification of electrospun fibrous scaffolds

3.1.1. Fabrication of electrospun fibrous scaffolds

8% solution of poly(lactic-co-glycolic)acid (PLGA) (Evonik Industries, Essen, Germany) was prepared by dissolving required amount of polymer granules in 7:3 (V:V) chloroform (CHCl₃) and Dimethylformamide (DMF). A homogeneous polymer solution was achieved after stirring the polymer-solvent mixture for 4 consecutive hours. NANON-01A (MECC, Fukuoka, Japan), electrospinning apparatus was used to prepare PLGA fibers by using a flow rate of 0.5 ml/h through a 13.4-gauge blunt needle over a voltage of 18 kV at a working distance of 6 cm. A collection bath of 7:3 (v:v) isopropyl alcohol to water with 0.05% Pluronic F-108 surfactant was used to collect the spun fibers. Metallic molds of defined dimensions were used to collect the fibers from the liquid collection bath followed by freeze-drying to retain the scaffold dimensions. For two dimensional electrospun fibrous mat, the same polymer solution was spun at a voltage of 20 kV with a working distance of 12 cm and collected on an aluminum foil. Residual solvent was removed by overnight incubation at 37°C in a vacuum oven.

3.1.2 Surface modification of electrospun scaffolds

N-ethyl-N'-(3-dimethyl aminopropyl) carbodiimide (EDC), N-hydroxy succinimide (NHS) technique was used to surface modify the electrospun PLGA fibers. Freeze-dried PLGA fiber surface was hydrolyzed over 30 mins with 0.01 N Sodium hydroxide (NaOH) solution at room temperature (RT). The hydrolyzing base solution was removed from the spun fibers by washing with DI water for 3 time. 50 mM 2-ethane sulfonic acid (MES) buffer solution (pH=6) with 80 mM NHS and 40 mM EDC was then added to the pre-hydrolyzed fiber surface and kept at 4°C for 4 hours. The unreacted chemical mixture was washed out with DI water. Activated spun fibers were chemically modified by treating with a single protein solution or multiple protein solutions and incubated at 4°C overnight under mild stirring. The unreacted protein solution was then removed by washing with DI water three times and kept at -80°C

followed by freeze-drying. This process is also known as chemisorption of electrospun fibers.

3.2 Characterization of electrospun fibers

3.2.1 Morphological analysis of electrospun fibers

Fiber morphology was analysed with Scanning electron microscopy (SEM, JEOL JSM-6340F). In SEM, the specimen surface was scanned with high energy electrons which gives rise to the inelastic emission of secondary electrons from the sample surface due to the exchange of energy between the sample and the electron beam. Thus, the better conductivity of the sample surface can improve the resolution of SEM images. However, due to the lower conductivity of biopolymers and biological specimens, the surface of the samples was gold-sputtered at 20 mA for 60 seconds. To avoid overcharging which may cause poor image resolution, a working voltage of 5 kV was applied for all the samples. Images were captured at different magnifications (500X; 1000X; 5000X). Pore size and diameter of both 2D and 3D electrospun fibers were measured for 100 different locations from five random images by using ImageJ software. The collected data was plotted as a histogram to visualize diameter and pore size distribution by using OriginPro 2016.

3.2.2 Chemical quantification of electrospun scaffolds

Fourier transform infrared spectroscopy (FTIR) was (Perkin Elmer Inc., Shelton, USA) used to analyze the chemical properties of the electrospun scaffolds. It is an energy absorption technique where samples were scanned with IR radiation over a range of wavelengths. Functional groups present in samples absorb beams at specific wavelengths which get recorded and analyzed. A strict sample drying process is needed to follow in this characterization technique to avoid moisture interference with other absorption bands. Freeze-dried samples were encapsulated in potassium bromide (KBr) pellets and scanned at a range of 200-4000 cm^{-1} to evaluate chemical characteristics of unmodified and surface modified electrospun fibers.

3.2.3 Protein quantification of ECM modified scaffolds

Bicinchoninic acid assay (BCA assay) was applied to quantify the amount of total amount of protein chemisorbed by surface-modified electrospun scaffolds. It is a colorimetric test where a sample solution changes its color from green to purple. The peptide bonds present in proteins reduce Cu^{2+} to Cu^+ ion. The amount of Cu^{2+} reduced by the peptides were quantified by measuring the light absorption of purple-colored bicinchoninic acid and Cu^+ chelate complex at 562 nm wavelength.

3.3 Cell culture study on electrospun scaffolds

3.3.1 Materials

Primary human hepatocytes (PHHs) were purchased from Yecuris Corporation, Portland, OR. Hepatocyte Culture Medium (HCM) was obtained from Lonza Walkersville Inc., Walkersville, MD. Human hepatic stellate cells (HSCs) were obtained from Samsara Sciences INC, San Diego, CA. LX-2 cells were provided by Dr. Jason A. Wertheim lab, Northwestern University, Chicago. Huh-7.5 cells were purchased from Apath, NY. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum albumin (FBS), 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco, Thermofisher Scientific, USA. Live-dead viability/cytotoxicity kit from Life Technologies, California, USA was used to visualize the cell viability. Trizol reagent was purchased from Amnion, CA. 4',6-diamidino-2-phenylindole (DAPI) and Hoechst 33342 were obtained from Thermo Fisher Scientific, USA. The primers used for gene expression studies were obtained from IDT, Singapore. All ELISA kits were purchased from abcam, Singapore. Before cell seeding, all 2D and 3D scaffolds were sterilized under UV at 254 nm wavelength followed by washing with 70% EtOH solution. The sterilized scaffolds were then washed with PBS (Phosphate-buffered saline) for three times, 5 mins each.

3.3.2 Cell culture

Primary human hepatocytes were cultured in hepatocytes culture media. LX-2 at passage 7 were maintained in DMEM media with 1% antibiotic (100 U/ml penicillin, 100 µg/ml streptomycin) and 2% FBS before co-culture with primary human hepatocytes. Primary human hepatic stellate cells (HSCs) were maintained in complete DMEM media until passage two before seeding on different substrates. For monoculture or co-culture of PHHs with LX-2 cells or HSCs the culture media was changed every day with fresh hepatocytes culture media. Huh-7.5 cells maintained in 1% antibiotics and 10% FBS containing DMEM media before seeding on scaffolds. The culture media was exchanged with fresh DMEM media in every 2-3 days. All the cultures were kept in 37°C humidified incubator with 5% CO₂. The cultures were detached by using 0.25% trypsin-EDTA solution after washing with 1x PBS for three times.

3.3.3 Quantitative polymerase chain reaction (qPCR)

The cells cultured on a 2D substrate (either tissue culture plate or in sandwich culture) were trypsinized and centrifuged before the RNA extraction process. For 3D cultures, after three times washing with PBS, the scaffolds with cells were completely dissolved in Trizol reagent. Trizol reagent (Amnion, CA) was used to extract the total RNA from cells on at pre-determined time points. The concentration of the extracted RNA in diethylpyrocarbonate (DEPC) water was measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific, US). Reverse transcription of the RNA solution was used to synthesize cDNA by using an iScript™ cDNA Synthesis Kit (Bio-Rad, California, US) and a 2720 thermal cycler (Applied biosystems, California, US). To prepare the cDNA, the reaction mixture was heated at 25°C for 5 minutes, followed by heating at 46°C for 20 minutes and finally 95°C for 1 minute. qRT-PCR was performed by using SYBR® Select Master Mix (Applied biosystems) and a CFX96™ Real-Time PCR Detection System (Bio-Rad). The thermal cycle used was: Heating at 50°C for 2 minutes, 95°C for 2 min followed by 39 times repetition of 0.15-minute heating at 95°C

and 1-minute heating at 60°C, Finally 0.05 minute heating at 65°C and 0.5 minute heating at 95°C. Gene expression values were normalized to an internal control/housekeeping gene and expressed as a fold change relative to the gene expression of respective controls by using the $\Delta\Delta CT$ method. The primer sequences used for each gene are listed in **Table 3.1**.

Table 3.1: List of primers

Target Gene	Forward (5'-3')	Reverse (5'-3')
Albumin (ALB)	CTGCACAGAATCCTTGGTGA	CTCCTTATCGTCAGCCTTGC
CYP3A4	ACCGTGACCCAAAGTACTGG	TTCAGGGGGATCTGTGTTTC
CYP3A7	AAGTGGACCCAGAAACTGCA	GGCTCCACTTACGGTCTCAT
CYP2C9	GGATTTGCCTCTGTGCCGCC	GCAGCCAGGCCATCTGCTCTT
CYP2E1	AATGGACCTACCTGGAAGGAC	CCTCTGGATCCG GCTCTCATT
CYP1A2	TGGCCTCTGCCATCTTCTG	GGACCCGAGGCCTCAAAC
E-Cadherin (E-Cad)	CGAGAGCTACACGTTACGG	GGGTGTCGAGGGAAAAATAGG
Cyclopontin	CACAGGAGGAAAGAGCATCTAC	CACAGACGGTCACTCAAAGAA
Collagen-1A1 (Col1A1)	TTCTGCAACATGGAGACTG	ATGTAGGCCACGCTGTTCT
Alpha-Smooth muscle actin (α-SMA)	AAAAGACAGCTACGTGGGTGA	GCCATGTTCTATCGGGTACTTC
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	CCATGGGGAAGGTGAAGGTC	CTCGCTCCTGGAAGATGGTG

3.3.4 Albumin secretion assay

Albumin secretion is a defining property of human hepatocytes. Albumin secretion assay was performed at defined time points by collecting culture media to analyze the synthetic efficiency of human hepatocytes. Human albumin ELISA kit (Abcam, Cambridge, United Kingdom) was used to quantify the albumin secretion from cell culture supernatant collected either after 24 hours incubation or 48 hours incubation.

3.3.5 Statistical analysis

A minimum of three samples had been used to conclude each result for experimental samples. All the data have been expressed as mean $\pm$ SD (standard deviation). Statistical

significance was examined by using two-way analysis of variance followed by Tukey testing between different groups. The null hypothesis considered was that the "samples have equal means". A p-value of <0.05 was considered as statistically significant when comparing between different groups of data.

Chapter 4

Analysis of translational and transcriptional properties of primary human hepatocytes cultured on ECM-modified 3D electrospun scaffolds

This chapter includes the fabrication and characterization of electrospun nanofibrous random PLGA scaffolds for maintenance of functional human hepatocytes in vitro. Two-dimensional (2D) and three-dimensional (3D) fibrous scaffolds were fabricated and characterized for culturing human liver cells. Gold standard sandwich culture and fresh primary human hepatocytes were used as controls. The effect of minimal essential ECM protein modifications into the random electrospun fibers on in vitro maintenance of functional hepatocytes was analyzed and discussed. A differential effect of two different ECM proteins on translational and transcriptional properties of hepatocytes was reported in this section which leads to a future direction of application of multiple proteins for scaffold surface modification and use of other liver cells along to enhance functional maintenance of primary hepatocytes in vitro. We have already published the work presented in this chapter [2].

4.1 Introduction

Hepatocytes comprise ~80% of the liver volume, drive significant roles in metabolic regulation, protein synthesis, detoxification and glycogen storage remain dispersed on a three-dimensional (3D) web-like structure of extracellular matrix which plays crucial role in functional maintenance and maturation of hepatocytes *in vivo*. Liver ECM consists of macromolecular proteins such as different types of collagens and fibronectins. These ECM macromolecules maintain genotypic and phenotypic characteristics of hepatocytes by providing matrix associated biochemical cues that becomes unavailable while conducting ex-vivo cultures of primary hepatocytes [145]. Thus, hepatocytes undergo dedifferentiation in very early stages of ex-vivo culture results progressive loss of liver-specific functional activities [113, 145]. This functional deterioration of primary hepatocytes *in vitro* cultures creating obstacles in cell-based therapy development or testing of drugs for liver diseases or even in studies on engineered liver tissues [146].

Among different technologies discussed previously by researchers, sandwich culture was reported as gold standard where hepatocytes were cultured in between layers of collagen and matrigel. This technology found to be beneficial for maintenance of functional hepatocytes compared to a single layer two-dimensional culture. However, the functions of hepatocytes decline gradually over time due to less 3D layering in sandwich cultures. Again, the use of matrigel for therapeutic models are less efficient due to its secretion by cancerous cell lines in non-uniform compositions [147-150]. This work is motivated by the need of developing a liver ECM mimicking scaffold with defined and essential macromolecular ECM proteins to support functions of human primary hepatocytes *in vitro*. Along with the chemical characteristics, a 3D structure with optimal porosity for better infiltration of hepatocytes to create *in vivo* microenvironment was found to be crucial.

Electrospinning is a very well-known technique to create micro to Nano-dimensional random fibers which can resemble the fibrous architecture of native liver ECM [151,

152]. The advantages of electrospinning include: 1) ease of fabrication of fibers from any soluble polymers; 2) simple processing technique; and 3) cost-effectiveness [153]. Due to these advantages, this technique has been used widely to produce scaffolds from many biopolymers either synthetic or natural for biomedical applications such as cardiac[154], neural[155], bone[156], and skin[157] tissue engineering. The major challenge of conventional electrospinning process is the closely packed fibers collected on a metallic collector with high fiber density which acts as a barrier for cell infiltration inside the scaffold [158]. To overcome this challenge, a liquid collecting technique, also known as wet electrospinning technique was developed where a fiber collection bath was used to prepare scaffolds with improved porosity and less fiber density [159]. This wet electrospinning technique gives a highly porous 3D spongiform scaffold which we found have a morphological similarity with native liver ECM matrix [160]. In this study, poly(lactic-co-glycolic) acid (PLGA) was used to fabricate 3D electrospun scaffolds followed by surface modification with biologically active ECM molecules to favor cell adhesion and to promote matrix associated cell signaling pathways for integrin-mediated binding and intercellular signaling. Collagen-I and fibronectin was chosen for surface modification of PLGA scaffolds due to the abundance of these two macromolecular proteins in liver extracellular matrix. Collagen-I plays a significant role in maturation of hepatocytes whereas fibronectin is an important mediator for integrin related cell signaling and attachment [161].

The objective of the proposed study was to establish a 3D nanofibrous biosynthetic hybrid material (PLGA fibers modified with ECM proteins) to maintain and enhance the functionality and viability of primary human hepatocytes *in vitro* which can further be used as a platform to scan new medications or use of functional hepatocytes in cell therapy-related applications. In comparison to the protein unmodified scaffolds or fibronectin modified scaffolds, collagen-I modified fibrous scaffolds were found to be most potent to maintain functional hepatocytes in this proposed *in vitro* model.

4.2 Materials and methods

4.2.1 Cell culture

Human primary hepatocytes have been expanded inside a $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ triple knock out mice (Yecuris Corporation, Portland, OR) and shipped fresh [162]. Hepatocyte Culture Medium (HCM, Lonza Walkersville Inc., Walkersville, MD) was used to maintain the freshly purchased hepatocytes *in vitro*.

4.2.2 Cell seeding

Scaffolds (diameter = 6 mm, thickness = 3 mm) were placed into the well of a 96-well plate. 40 μ L cell suspension (10,000 cells/ μ L) of hepatocytes was seeded onto each scaffold. A rocker plate was used to incubate the culture plate for 20 minutes for allowing homogeneous distribution and attachment of the cells on the scaffold. Another 160 μ L of culture medium was added to each well for making the total working volume to 200 μ L. Cultures were maintained at 37 °C and 5% CO₂ in a cell incubator with a humidified atmosphere. Daily change of media was performed. Sandwich culture of primary human hepatocytes were established by seeding 100 μ L of hepatocytes (1,000 cells/ μ L) on collagen-I coated culture plate and after overnight incubation, a layer of Matrigel was added upon the cells by adding 0.25 mg/mL growth factor reduced Matrigel Matrix (Corning). Sandwich cultures were maintained at 37 °C and 5% CO₂ in a cell incubator with a humidified atmosphere.

4.2.3 Resazurin Assay

Cell number at different time points was measured by using resazurin assay, which was described elsewhere [163]. Briefly, resazurin sodium salt (Sigma, USA) from the 44 μ M stock solution was diluted with HCM in 1:10 ratio to prepare fresh resazurin working solution on day 2, 7, 11 and 14. 100 μ L working solution was added to each well after removing the culture media and incubate for 1 hr. at similar culture

conditions. After the incubation, the partially reduced resazurin solution was removed to a black, opaque 96-well plate and spectrophotometer (Cytation 3, BioTek Instruments, Inc.) was used to measure the fluorescence at excitation: 540 nm; emission: 590 nm. As a positive control, completely reduced resazurin solution was used. Fresh primary hepatocytes seeded in a serially diluted cell suspension was used to prepared standard curve which presents a relationship between cell number with the absorbance presented as: volume ratio (10^6 cells/mL) to the measured fluorescence intensity (FI). This standard equation obtain from the standard plot was used to calculate the cell number at each time point by using resazurin assay.

4.2.4 Hematoxylin and eosin staining (H&E staining) and SEM analysis of cells on scaffolds

Wet electrospun 3D PLGA scaffolds with primary hepatocytes were fixed with 10% formalin solution on day 14. The fixed cultures were embedded and processed with paraffin and cut into 5 μ m thick sections. All sections were stained with hematoxylin and eosin (H&E).

SEM analysis was performed on day 14. Samples were fixed with 2.5% glutaraldehyde in PBS at 4 °C overnight. Fixed samples were then washed with PBS and treated with 1% osmium tetroxide for 1 hr. A series of dehydration was performed with the fixed samples starting from 25%, 50%, 75%, 95% EtOH for 15 minutes each and finally with 100% EtOH for 3 times, 15 minutes each. Samdri®-795 CO₂ critical point dryer (Tousimis, Rockville, MD, USA) was used to dry the samples, followed by osmium coating with OPC-60A Osmium Plasma Coater. Hitachi S-3400N-II (Hitachi High Technologies America, Inc.) SEM instrument was used to visualize the morphology of the primary hepatocytes cultured on unmodified PLGA and PLGA-ECM scaffolds.

4.2.5 Albumin secretion assay

Human ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) was used to measure albumin secretion on day 2, 7, 11 and 14. Samples collected for the assay was stored at -20 °C until used.

4.2.6 Urea secretion assay

Urea assay kit (Abcam, Cambridge, MA) was used to quantify urea secretion on day 2, 7, 11 and 14 to measure metabolic functions of primary hepatocytes. Samples collected for the assay was stored at -20 °C until used.

4.2.7 Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

qRT-PCR was applied to measure the liver-specific gene expressions of PHHs on day 14 in the 3D cultures and for the sandwich culture. TRIzol™ Reagent (Thermo Fisher Scientific Life Sciences, USA) was used to extract the RNA and treated with DNase (DNA-free™ Kit, Thermo Fisher Scientific Life Sciences, USA). iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used to perform reverse transcription reaction. qRT-PCR was performed on QuantStudio™ 7 System (Thermo Fisher Scientific Life Sciences, USA) by using SYBR Green PCR (Bio-Rad Laboratories, USA). $\Delta\Delta CT$ method was used to evaluate Gene transcription and normalized with to fresh (day 0) primary hepatocyte gene expression data and sandwich culture gene expression data. Cyclophilin was used as a housekeeping gene.

4.2.8 Quantification of cytochrome P450 enzyme activities for CYP2C9 and CYP3A4

3D cultures of primary hepatocytes and sandwich cultures were treated with or without an induction agent (25 μ M rifampicin) for 48 h prior to the P450 activity analysis. CYP

metabolites in the culture media was measured with Promega P450 bioluminescence enzymatic assays [164, 165]. Subsequently, cells were incubated for 4 hrs. with 100 μ M Luciferin-H (CYP2C9) and 1 hr. with 3 μ M Luciferin-IPA (CYP3A4) (Promega, Madison, WI) at 37 °C. After the incubation, 25 μ L luciferin medium was added to 96-well white opaque plate and treated with 25 μ L the luciferin detection reagent. A Synergy2 microplate reader (BioTek, USA) was used to measure luminescence signal. Cell number was measured by using resazurin assay after measuring the CYP activity.

4.3 Results

4.3.1 Preparation and Characterization of PLGA Scaffolds

Nanofibrous PLGA scaffolds, prepared by wet electrospinning process was compared with the nanofibrous sheet prepared by conventional electrospinning process. The SEM images captured of each scaffold were used to calculate the fiber diameter and porosity. A higher degree of compact layering was evident in the fiber mat produced by conventional electrospinning process whereas a less compact and more porous structure was achieved by using wet electrospinning process in the 3D fibrous scaffolds (**Figure 4.1A, B**).

Figure 4.1: Conventional and wet electrospinning process: A) SEM images of a nanofibrous electrospun scaffold sheet collected from the aluminum foil with a smaller pore size compared to B) Larger pores observed in scaffolds prepared by wet electrospinning. Scale bar = 10 μm; Magnification = 1000x.

In 2D fibrous mat the pore size achieved was 5.2 to 19.7 μm which is much smaller than the size of the primary human hepatocytes (15-30 μm) [166]. 3D PLGA scaffolds obtain by liquid collection technique gives a pore size in the range of 9.7 to 44.5 (**Figure 4.1D**) with an average pore size of $27.5 \pm 7.5 \mu\text{m}$ ($p < 0.001$ compared to the pore size obtain by traditional technique, **Figure 4.1C**), which is suitable for better infiltration of primary human hepatocytes inside the scaffolds as the decellularized human liver is found to have a pore size of ~30 μm [110]. However, the average diameter obtained by wet electrospinning technique ($870 \pm 300 \text{ nm}$, range 500 to 1,400 nm, (**Figure 4.2**) was remain indifferent ($p = 0.07$) compared to the fiber diameter obtain by traditional electrospinning technique ($1,000 \pm 250 \text{ nm}$).

Figure 4.2: Diameter distribution of the fibers within wet electrospun PLGA nanofibers scaffolds; $n = 3$.

The pore sizes of the scaffolds obtain by two different techniques were further tracked by using growth and infiltration of the primary hepatocytes into the scaffolds. A 2- to 4 – fold higher cell number ($p < 0.05$, $p < 0.01$) was remained after 2 days of culture in the 3D wet electrospun scaffolds due to improved pore size compared to the conventionally electrospun scaffolds (**Figure 4.3A**).

Initially, fresh primary human hepatocytes (PHHs) were cultured on 2D PLGA fibrous mat which was unmodified or modified with $100 \mu\text{l/ml}$ collagen-I (PLGA-C_{high}).

Figure 4.3: Top row: (A) Metabolic activity of viable human primary hepatocytes on day 7 within 2D electrospun fibrous sheet, on unmodified wet electrospun fibers and on PLGA-C_{high} scaffolds. Cell viability was determined by using resazurin assay on day 2, 4 and 7 (n = 6 for each group, *p < 0.05, **p < 0.01)

Bottom row: (B) SEM images of human primary hepatocytes on conventional electrospun scaffolds and within (C) PLGA-C_{high} are presented on day 2 (500X, scale bar = 50 μm). Insets: Cross-sectional view of hepatocytes stained with hematoxylin and eosin at 40X, scalebar = 20 μm.

Due to smaller pore size in the later, H&E-staining of scaffold cross-section showed no penetration of the cells through the fiber mat and it remains only on the scaffold surface (Figure 4.3C).

Taken together, the conventional electrospinning process results close-packed fiber layer which severely limits cell infiltration due to smaller pore sizes and higher density of fibers. Thus, to develop scaffolds with essential ECM proteins for 3D culture of primary human hepatocytes, 3D electrospun scaffolds obtained by wet electrospinning technique because of its highly porous (>90%) well connected structure for cell infiltration and growth.

4.3.2 Immobilization of ECM Proteins on Electrospun PLGA Scaffolds

Two different concentrations of collagen-I and fibronectin (100 µg and 50 µg for collagen-I; 50 µg and 25 µg for fibronectin) was used to chemically modify the PLGA fiber surface by using EDC/NHS technique [167]. The retention of scaffolds porosity and 3D structure were ensured by using SEM imaging. All the PLGA 3D scaffolds, irrespective of the type of ECM modification were able to maintain the pore size and porosity in both vertical and horizontal direction was conformed from the SEM images (**Figure 4.4A-D**).

Figure 4.4: SEM images of nanofibrous scaffolds modified with collagen-I (A) 100 $\mu\text{g/mL}$ and (B) 50 $\mu\text{g/mL}$ (Top row) ; with Fibronectin (C) 50 $\mu\text{g/mL}$ and (D) 25 $\mu\text{g/mL}$ (Bottom row). Scale bar = 10 μm ; Magnification = 1000x.

The presence of ECM proteins on fiber surface after chemisorption was confirmed by using FTIR analysis. In protein modified scaffolds, peaks at 1637 cm^{-1} , 1562 cm^{-1} , and 1240 cm^{-1} corresponding to N-H bending of primary amine was observed (**Figure 4.5A**), which was absent in unmodified PLGA scaffolds indicating the presence of proteins conjugated to PLGA fiber. The total protein chemisorbed on PLGA fibers were measured by using BCA assay. In collagen-I modified scaffolds the range of total protein content was $4.4 \pm 0.3\text{ }\mu\text{g/mg}$ for PLGA- C_{high} and $2.4 \pm 0.5\text{ }\mu\text{g/mg}$ for PLGA- C_{low} whereas for fibronectin modified scaffolds, it was $1.4 \pm 0.5\text{ }\mu\text{g/mg}$ for PLGA- F_{high} and $0.9 \pm 0.4\text{ }\mu\text{g/mg}$ for PLGA- F_{low} (**Figure 4.5B**). All modified and chemically surface modified PLGA scaffolds prepared by wet electrospinning process found to have equivalent porosity, pore size and fiber diameters.

Figure 4.5: Chemical analysis of ECM-modified and unmodified PLGA scaffolds. (A) ATR-FTIR analysis gives peaks at 1637 cm⁻¹, 1562 cm⁻¹, and 1240 cm⁻¹ for ECM modified scaffolds, was absent for unmodified PLGA scaffold; n = 3. (B) Pore size, fiber diameter and porosity of the scaffold were quantified for protein modified and unmodified scaffolds; n = 3.

4.3.3 Primary Hepatocyte Culture within Wet Electrospun PLGA-ECM Scaffolds

A 14-days cell culture experiment was carried out on ECM-modified 3D electrospun PLGA scaffolds to study the maintenance of functionality and viability of primary human hepatocytes. Resazurin assay, a non-invasive method was used to measure the viability of PHHs at day 2, 7, 11 and 14 (**Figure 4.6**) by using calorimetric quantification of the degree of reduction in resazurin inside cell mitochondria [163]. The change in

color of culture media due to resazurin reduction was quantified by using a spectrophotometer without causing any disturbance to the growing cells. Terminally differentiated primary hepatocytes rarely proliferate in *in-vitro* cultures and the aim of the serial determination of cell viability at different times points was to shown the non-inferiority of ECM modified 3D electrospun scaffolds in maintenance of number of viable cells in comparison to the unmodified 3D PLGA scaffolds and gold standard sandwich culture.

Figure 4.6: Number of viable cells were determined by using resazurin assay on days 2, 7, 11, and 14, (n = 6 for each group), **p < 0.01, ***p < 0.001.

On day 2, significantly higher number of cells was present on PLGA-C_{high}, PLGA-C_{low}, and PLGA-F_{low} scaffolds ($3.11 \pm 0.62 \times 10^4$, $2.75 \pm 0.64 \times 10^4$, and $2.43 \pm 0.88 \times 10^4$ cells per scaffold, respectively) when compared to the unmodified 3D PLGA scaffolds and PLGA-F_{high} scaffolds ($1.64 \pm 0.04 \times 10^4$ and $1.68 \pm 0.38 \times 10^4$ cells per scaffold). Second, cell enumeration indicates that an initial drop of cell number happens between day 2 to days 7 in all scaffolds (60% cells remaining in PLGA-C_{high}, 58% cells remaining in PLGA-C_{low}, 83% cells remaining in PLGA-F_{high}, 63% cells remaining in PLGA-F_{low}, and 77% cells remaining in PLGA). The cell viability was maintained

during extended culture to 14 days on all scaffold types (for day 7 to day 14: PLGA- C_{low} $p = 0.24$; PLGA- F_{high} $p = 0.15$; for day 11 to day 14: PLGA- C_{high} $p = 0.51$; PLGA- F_{low} $p = 0.21$; PLGA $p = 0.27$). The cell number in collagen I/Matrigel sandwich culture remain unaffected though out the 14 days of culture. The probable cause of decrease in total cell number from day 2 to day 7 in 3D electrospun scaffolds may be attributed by the fall of non-adherent cells from the scaffolds to the non-adherent wells and removed overtime during change of cell culture media. This phenomenon was absent for the sandwich culture where the hepatocytes adhered on the collagen-I coated wells and further covered with the Matrigel coating. Nonetheless, the cell number in collagen-I/matrigel sandwich culture remain largely comparable to the cell number quantified on PLGA-based 3D scaffolds, thus, demonstrated the equivalency at the termination of 14 days cell culture study.

The morphology of primary human hepatocytes was visualized by using SEM images after 14 days of cell culture on 3D PLGA scaffolds. Clusters of primary hepatocytes with good cell-cell contact was observed in protein modified PLGA scaffolds and was absent in unmodified 3D PLGA scaffolds **Figure 4.7**. Some of the cells acquires a more rounded shape may be due to its differentiated phenotype [168, 169]. On ECM modified scaffolds, more particularly on collagen-I modified scaffolds, hepatocytes grown and spread along and across the fibers, also wrap around the nanofibers of PLGA- C_{high} scaffold (**Figure 4.7**). H&E staining of the scaffolds after day 14 again confirms the cell spreading along the nanofibrous PLGA- C_{high} scaffolds (**Figure 4.7B, inset**).

Figure 4.7: Attachment of primary human hepatocytes on unmodified and ECM-modified PLGA fibers were visualized by using SEM micrographs (1000X, scale bar = 50 μm). A) unmodified PLGA, B) PLGA-C_{high}, C) PLGA-C_{low}, D) PLGA-F_{high}, E) PLGA-F_{low}. Insets: Cross-sectional view of same cells on PLGA scaffolds where cells stained with hematoxylin and eosin (at 63X, scale bar = 20 μm)

Notably, the SEM images confirm the better cell abundance in protein modified scaffolds thus, indicating the role of biologically active protein molecules in attachment and viability of primary hepatocytes for *in vitro* cultures. Hepatocytes can establish cell-cell contacts also on the mono-layer sandwich culture (**Figure 4.8**).

Figure 4.8: H&E-staining (20X) of primary human hepatocytes on day 7 within collagen-Matrigel sandwich culture; scale bar = 100 μ m.

However, this culture lacks the 3D fibrous network structure provided by the 3D electrospun scaffolds. Thus, in comparison to gold standard sandwich culture, the reported cell-laden scaffolds showed cell penetration inside the 3D matrix visualized by H&E staining. Again, ECM modification on PLGA scaffolds further improved the cell-cell and cell-matrix interactions compared to the unmodified PLGA scaffolds. Among all the ECM modified PLGA scaffolds, PLGA-C_{high} showed the highest cell-cell and cell-matrix interactions (**Figure 4.7b**).

4.3.4 Expressions of liver specific genes

qRT-PCR was used to enumerate the transcription levels of liver-specific genes after 14 days culture of primary human hepatocytes within different culture configurations. After normalizing against the housekeeping gene, all the gene expressions were further normalized over the transcription level in fresh primary hepatocytes or over day 14 sandwich culture (**Figure 4.9**). The mRNA expression of albumin, which also regulates the synthetic functions of hepatocytes found to be maximum for the cells cultured on PLGA-C_{high} scaffolds, compared to both the controls (**Figure 4.9 A,B**).

(A)

(B)

Figure 4.9: On day 14, albumin, CYP2C9, and CYP3A4 gene expressions of primary human hepatocytes (PHHs) cultured within each scaffold configurations were quantified. A) Sandwich culture used as a control and results were normalized over the control (n = 3 for each culture group), *p < 0.05, **p < 0.01, # indicating unevaluated statistics due to the lack of expression, and B) Fresh (day = 0) human primary hepatocytes applied as a control for imitating the ideal *in vivo* hepatocyte function, (n = 3), *p < 0.05, **p < 0.01, ***p < 0.001, ND = not detected.; # = statistics not able to be calculated due to lack of PLGA expression.

Primary human hepatocytes cultured on PLGA-C_{high} scaffolds showed 3.5-fold higher albumin gene expression when compared to the 3D unmodified PLGA scaffolds (p <

0.001). This observation indicates the importance of collagen-I presence on the scaffold for regulation of liver-specific gene expressions of human primary hepatocytes. PLGA- C_{high} culture group again shown 4-fold higher expression of albumin ($p < 0.01$, **Figure 4.9B**) when compared to the fresh primary human hepatocytes (day 0) and 1.5-fold higher expression compared to the sandwich culture ($p < 0.01$, **Figure 4.9A**). In PLGA- F_{low} culture group, albumin expression improved by ~1.5-fold when compared to the sandwich culture at the end of 14 days.

Expressions of cytochrome P450 genes were investigated to determine the effect of different scaffold configurations modified with minimal essential ECM proteins at the transcriptional level of these catalytic enzymes. In the liver approximately 50% of the drugs get cleared by CYP3A4, a cytochrome P450 enzyme [170]. Thus, CYP3A4 gene expression level was determined for all culture types at day 14. An amount of total protein chemisorbed on PLGA- C_{high} and PLGA- C_{low} culture groups was found to affect the transcriptional level of CYP3A4 gene. On the contrary, CYP3A4 expression was undetectable on unmodified PLGA scaffolds along with the scaffolds modified with fibronectin i.e. PLGA- F_{high} and PLGA-Flow (**Figure 4.9 A,B**). A 4-fold higher CYP3A4 expression of hepatocytes have been quantified for the cells cultured on PLGA- C_{high} scaffolds ($p < 0.01$, **Figure 4.9A**) when compared to the gold standard sandwich culture. Furthermore, compared to the fresh primary hepatocytes, PLGA- C_{high} and PLGA- C_{low} registered 1.6-fold and 1.5-fold higher expressions respectively after 14 days of culture (**Figure 4.9B**).

CYP2C9, another important member of cytochrome P450 family, catalyzed warfarin, also shown highest expression on PLGA- C_{high} scaffolds on day 14 among all the culture groups. A 3-fold high expression of CYP2C9 was found in PLGA- C_{high} scaffolds when compared to the hepatocytes cultured in the mono layer sandwich culture ($p = 0.019$) (**Figure 4.9A,B**). However, the expression of CYP2C9 remain undetectable for the hepatocytes cultured on unmodified PLGA scaffolds. Taken together, both catalytic and synthetic gene expressions strongly suggest that collagen-I modification of 3D

electrospun scaffold can improve the cell microenvironment of hepatocytes, thus stimulate liver specific gene expressions at the transcriptional level.

4.3.5 Quantification of urea and albumin secretion of primary human hepatocytes on wet electrospun 3D fibrous scaffolds

Synthetic functions of primary hepatocytes cultured on different scaffolds were quantified by evaluating the amount of albumin and urea secretion by the cells at different time points after normalizing with the cell numbers on the day of sampling over 14 days of culture. After 48 hrs. of culture the albumin and urea production were quantified and established as baseline production. The albumin secretion by primary human hepatocytes on PLGA-C_{high} scaffolds increased from 24.36 ± 4.35 $\mu\text{g}/10^6$ cells/day on day 2 to 34.56 ± 2.54 $\mu\text{g}/10^6$ cells/day on day 14 ($p = 0.044$, **Figure 4.10A**).

(A)

(B)

Figure 4.10: Albumin and urea production by primary human hepatocytes over 14-day experiment was quantified for all cultures of 3D PLGA scaffolds and within sandwich culture. A) Albumin secretion was quantified on day 2, 7, 11, and 14 ($n = 4$ for each scaffold type). B) Urea synthesis was quantified on day 2, 7, 11, and 14 within 3D scaffolds and control. ($n = 3$ for each sample); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Conversely, in case of unmodified 3D PLGA scaffolds, the albumin secretion was decreased from $7.06 \pm 2.34 \mu\text{g}/10^6 \text{ cells/day}$ on day 2 to $3.27 \pm 0.83 \mu\text{g}/10^6 \text{ cells/day}$

on day 14 ($p = 0.02$). Primary human hepatocytes cultured on unmodified 3D PLGA scaffolds showed a constant lower production of albumin at all the measured time points when compared to the PLGA-C_{high} scaffolds ($p < 0.001$). At the end of 14 days the albumin production by hepatocytes within PLGA-C_{high} scaffolds ($34.56 \pm 2.54 \mu\text{g}/10^6$ cells/day) was significantly higher compared to the albumin produced by hepatocytes within sandwich culture ($11.59 \pm 3.07 \mu\text{g}/10^6$ cells/day, $p < 0.01$).

A similar observation was experienced for urea secretion by primary human hepatocytes. On PLGA-C_{high} scaffolds the urea secretion on day 14 was $49.68 \pm 1.63 \mu\text{g}/10^6$ cells/day, which was significantly higher compared to the urea secretion by hepatocytes on unmodified 3D PLGA scaffolds ($12.32 \pm 5.8 \mu\text{g}/10^6$ cells/day at day 14, $p < 0.001$, **Figure 4.10B**). The urea secretion on unmodified PLGA scaffolds at the end of 14 days was lowest compared to all other culture groups. Additionally, a higher production of urea by hepatocytes cultured on PLGA-C_{high} scaffolds was also observed on day 14 when compared to the collagen-I/ Matrigel sandwich culture ($20.49 \pm 2.31 \mu\text{g}/10^6$ cells/day, $p < 0.01$, **Figure 4.10B**). These results indicate the effect of cell microenvironment in improving and maintaining the liver specific functions of primary human hepatocytes on 3D electrospun ECM protein modified PLGA scaffolds over collagen-I/Matrigel sandwich culture. Additionally, the improvement in synthetic properties of hepatocytes on PLGA-C_{high} scaffolds further reinforces the observations acquired from the measurement of gene transcription demonstrating again the importance of collagen-I modification on 3D scaffolds.

4.3.6 Quantification of CYP activity

The catalytic functions of primary hepatocytes were further gauged by measuring the activity of CYP3A4 and CYP2C9 at day 7 and day 14 (**Figure 4.11**). Cytochrome P450 activities were measured in presence and absence of rifampicin, a drug extensively used to induce catalytic activity of CYP2C9 and CYP3A4 which defines specific characteristics of primary hepatocytes. CYP activities of fresh primary hepatocytes was measured as a control to imitate the standard *in vivo* fraction of primary hepatocytes. A

common trait of human primary hepatocytes is the gradual reduction of CYP P450 activity over time when cultured ex-vivo. This was evident from the gradual decrease of CYP catalytic activity in sandwich culture from day 7 to day 14, recovered to an extent after pharmacologic induction [171, 172]. Both CYP3A4 and CYP2C9 activities of primary hepatocytes likewise reduced from day 7 to 14 for the cells cultured on all ECM modified 3D scaffolds, including those cultured on Collagen-I/Matrigel sandwich culture (**Figure 4.11**).

Figure 4.11: Cytochrome P450 activities of human primary hepatocytes within each 3D PLGA scaffold and in sandwich culture. CYP2C9 and CYP3A4 activity at day 7 (A and B respectively) and CYP2C9 and CYP3A4 activity at day 14 (C and D respectively), with and without

induction by rifampicin is reported. Fresh (day 0) human hepatocytes were included as a control. (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001. ND = not detected.

However, the hepatocytes within PLGA-C_{high} scaffolds maintains higher activity of CYP2C9 (day 7 induced, p < 0.001; day 14: PLGA undetectable) and CYP3A4 (day 7 induced: p < 0.01; day 14 induced: p < 0.01) on day 7 to day 14 in comparison with the unmodified PLGA scaffolds, thus demonstrating the importance of collagen-I to potentiate the catalytic activity of primary hepatocytes in long-term *in vitro* culture. In comparison to sandwich culture, the uninduced activity of CYP2C9 on PLGA-C_{high} scaffolds was found to be equivalent on day 7 (p = 0.96). Activity of CYP2C9, induced pharmacologically with rifampicin in primary human hepatocytes within PLGA-C_{high} scaffolds were equivalent to the CYP2CP activity observed in sandwich culture both on day 7 (Figure 11a, p = 0.91) and day 14 (**Figure 4.11B**, p = 0.88).

Rifampicin induced CYP3A4 activity on day 7 and day 14 was higher for all culture conditions compared to the non-induced activity (**Figure 4.11 B,D**), which was largely undetectable by 14 days in PLGA-ECM scaffolds and negligible within the ECM sandwich control. Pharmacologic induction of P450 activity is a specific trait of primary hepatocytes, and is similar to the trend seen by others in which induction led to higher P450 activity.[173] Induced CYP3A4 activity was greatest in the PLGA-C_{high} scaffolds compared to all other PLGA scaffolds at both day 7 and 14 (**Figure 4.11C,D**), again showing the importance of type I collagen as a critical ECM constituent.

4.4 Discussion

In this study the development of a 3D electrospun synthetic PLGA fibrous scaffolds with minimal essential ECM protein modifications to mimic liver microenvironment was outlined for *in vitro* maintenance of functional primary human hepatocytes. The ability to fabricate scaffolds, chemically engrafted with ECM proteins or defined combinations of proteins may widen the *in vitro* culture of primary hepatocytes and

thus, may potentially enable the use of these cell-matrix combinations for clinical cellular therapies.

The 3D fibrous scaffolds fabricated, resemble the pore size of *in vivo* liver matrix, contain crucial components of the native liver microenvironment, and are synthetic, optimizable and easy to fabricate culture system to maintain human primary hepatocytes *in vitro*. To investigate important ECM proteins for these 3D PLGA-based fibrous matrices, the scaffolds were fabricated by using a wet electrospinning method that gives a highly porous, fibrous structure which closely replicates the porous, fibrous architecture of decellularized liver parenchyma. Amine coupling method was used to link collagen-I and fibronectin, two important ECM proteins to the fibrous scaffolds. The synthetic properties of hepatocytes (albumin and urea production) remain preserved on these scaffolds over 2 weeks of culture period. Moreover, unlike unmodified PLGA scaffolds collagen-I modified 3D electrospun scaffolds maintain and potentiates functions of primary hepatocytes. It was found that type-I collagen modified PLGA 3D scaffolds can exhibit better maintenance of synthetic and inducible catalytic activities of primary hepatocytes, thus indicating the critical requirement of collagen-I in liver microenvironment to influence hepatocytes functions. Additionally, primary hepatocytes cultured in PLGA-C_{high} scaffolds showed better synthetic and catalytic properties when compared to the fibronectin modified or unmodified PLGA 3D scaffolds. PLGA-C_{high} scaffolds also able to maintain higher transcriptional level of liver specific genes, along with the secretion of higher level of albumin and urea when compared to the hepatocytes cultured on gold standard sandwich culture. Taken together, these observations explain the benefit of using highly porous 3D electrospun scaffolds modified with collagen-I over collagen Matrigel sandwich culture to regulate functions of primary hepatocytes *in vitro*.

Previously, it was demonstrated that the application of this new wet electrospinning technique to produce polymeric scaffolds for healing chronic wounds [167]. In this study, a similar approach was used to fabricate a liver ECM mimicking scaffold with larger pores compared to the pore size obtain by conventional electrospinning process,

enables infiltration of primary hepatocytes inside the matrix. Electrospinning as a general method, have been used to fabricate scaffolds for liver tissue engineering has not been applied as a method to replicate porosity of native liver matrix in a synthetic scaffold. Rajendran et. al. recently reported the synthesis of fibronectin modified chitosan nanofibers by using conventional electrospinning technique to culture hepatocytes from rodent origin [174]. They reported rapid loss of albumin secretin of rat primary hepatocytes in contrast to the results presented in this section where it was found that PLGA- F_{low} secretes equivalent amount of albumin in comparison to collagen-I/Matrigel sandwich culture over 14 days of culture. In combination with the variation in cell source (rat) and type of biomaterial used, this study has shown the fabrication of larger pore by using wet electrospinning technique. The pore size reported by Rajendran et. al. study was smaller ($2.2 \pm 0.4 \mu\text{m}$ [174]) compared to the pore size obtain by using wet electrospinning technique ($27.5 \pm 7.5 \mu\text{m}$) as described here. A higher cell-cell/ cell matrix contact and cell infiltration were achieved as evident from SEM images and H&E staining of hepatocytes in the collagen-modified 3D wet electrospun PLGA scaffolds due to low fiber density which results in higher porosity (**Figure 4.7**).

Among different ECM proteins present in the liver, collagen-I is the most abundant, thus found to be critical for *in vitro* maintenance of functional hepatocytes. The findings strengthen the observations reported by other groups using different scaffolding systems for culture of primary hepatocytes. Kim et al. reported the fabrication of poly(ethylene glycol, PEG)-based 3D hydrogels modified with collagen-I to investigate its effect on Huh-7.5 cells, an immortalized cell line derived from human hepatocytes [175]. The presence of collagen-I on this hydrogel scaffolds leads to 4-fold improvement in albumin production after 14 days of culture compared to the bare scaffolds, demonstrating a similar observation to our results using human primary hepatocytes. An increase in liver specific gene expressions was also observed for collagen modified PEG hydrogel scaffolds compared to the unmodified scaffolds in Huh-7.5 cells. HepG2, Huh7 is very frequently used hepatoma-derived cell lines for *in vitro* studies due their easy availability, better stability in *in vitro* culture conditions

compared to the primary human hepatocytes which are very fragile and show greater susceptibility towards dedifferentiation. However, primary hepatocytes are the gold standard for *in vitro* studies on liver tissue engineering because they are not altered by any genetic mutations which can lead to cancer and they are the actual liver parenchymal cells that catalyze exogenous medications [176]. Another group Li et al. used PLGA scaffolds to culture fresh human primary hepatocytes [177]. The freeze-extraction process was used to add collagen-I on the PLGA scaffolds which were completely different from our methodology. An increase in albumin secretion of 1.63 ± 0.23 pg/cell/h (39.12 ± 5.52 μ g/ 10^6 cells/day) in collagen-I/PLGA scaffolds over 13 days of culture was reported over the unmodified scaffolds, which supports our observation for PLGA-C_{high} scaffolds after 14 days of culture (34.56 ± 2.54 μ g/ 10^6 cells/day). This level of albumin secretion was higher in comparison to the *in vivo* albumin production of 0.742 pg/cell/h (17.81 μ g/ 10^6 cells/day) further demonstrating the preservation hepato-specific functions [178-180].

Fibronectin is another important ECM protein that showed a beneficial effect on the *in vitro* growth of primary hepatocytes by promoting hepatocyte adhesion and regulation of hepato-specific genes [181-183]. Our findings revealed that the presence of fibronectin on the 3d fibrous PLGA scaffold is beneficial compared to the unmodified PLGA scaffolds in terms of increased albumin and urea secretion and improved gene expressions. Nonetheless, collagen-I modified fibrous scaffolds were superior to the fibronectin modified scaffolds at both concentrations, tested in all indices including albumin and urea secretions, gene expression analysis and cytochrome P450 activities [184].

Wang et al. demonstrated positive effects of fibronectin coating on an inverted colloidal crystal (ICC) scaffolds over bare scaffolds that did not modify with any biologically active ECM molecules [185]. In this study a ~80% higher secretion of albumin from Huh-7.5 cells cultured on fibronectin modified scaffolds was reported compared to the collagen-I modified scaffolds is contrasting our result, which may be attributed to the difference in cell type (Huh-7.5 cells) used to evaluate the performance of the 3D ICC

hydrogel scaffolds [176]. The same group also reported another study stating the co-presence of collagen-I and fibronectin on 3D ICC hydrogel scaffolds results improved liver-specific gene expressions (albumin, HNF4A) and albumin secretion compared to scaffolds modified with only collagen-I [186].

In this section a proof-of-concept was presented for application of 3D electrospun nanofibrous PLGA-ECM scaffolds as an *in vitro* culture model for functional primary hepatocytes. The positive effects of collagen-I modification on nanofibrous polymeric scaffolds in the maintenance of liver-specific functions of primary human hepatocytes was also established in this section. However, the proposed system also showed limitation in maintaining the primary hepatocytes cytochrome P450 activities which shown constant decay from day 7 to day 14 on ECM modified PLGA scaffolds as it was experienced in gold standard sandwich cultures. Nonetheless, the observations of this study indicate the important contributions of collagen-I to maintain synthetic properties of primary hepatocytes and make-up one of the most important ECM-component that must be retained in *in vitro* culture models to enhance functional maintenance of primary hepatocytes.

4.5 Conclusions

The work reported here elaborates that collagen-I modified wet electrospun PLGA nanofibrous 3D scaffolds augment human primary hepatocytes synthetic activities, thus may serve as a bioengineered starting material for the long-term maintenance of hepatocytes *in vitro*. After modification with biologically active macromolecules, the highly porous scaffolds developed by the wet electrospinning process mimics the native ECM structure, which is an essential scaffold characteristic for adhesion and functions of hepatocytes. The highly porous micro-environment is absent completely in sandwich culture, which is restricted by its synthesis in transformed cells and may have inconsistent protein compositions. The incorporation of minimal essential ECM proteins on the surface of biosynthetic scaffolds was presented, which leads to enhanced *in vitro* maintenance of human primary hepatocytes stimulated mainly by collagen-I. In

the next chapters, a combination of two ECM proteins along with the combination of two different liver cells is presented for investigating hepatic functions of primary hepatocytes for biomedical applications e.g. disease [187] based drug testing, toxicological screening and human cellular therapies.

Chapter 5

Co-culture of primary human hepatocytes with hepatic stellate cells on collagen-I modified 3D nanofibrous scaffolds to enhance *in vitro* maintenance of primary human hepatocytes

The effect of the co-culture of primary human hepatocytes with human hepatic stellate cells on collagen-I modified 3D nanofibrous PLGA scaffolds is discussed in this chapter. The optimal scaffold configuration discussed in chapter 4 was used in this work to optimize the ratio of PHHs to HSCs to establish an in-vitro model for liver tissue engineering. The optimization was performed based on cell viability, liver specific gene activity, albumin synthesis by PHHs and finally by analyzing the enzymatic activities of PHHs on different culture conditions. The results were further discussed and concluded based on the experimental observations.

5.1 Introduction

Hepatocytes hardly retain their liver-specific functional properties and undergo phenotypic changes in *in-vitro* culture models, thus posing a great obstacle for fabrication of *ex-vivo* models for drug screening, toxicity testing, or in developing hepatocytes-based therapies [145, 168]. To reconstruct cellular polarity, double layer sandwich culture [149, 188, 189] and spheroid cultures [190-192] were developed and have resulted in several successes in *ex-vivo* maintenance of hepatocytes functions when compared to hepatocytes monocultures. However, studies reported lack of expressions in certain differentiated functions, translational and transcriptional properties, or maintenance of cytochrome P450 enzymatic activities have necessitated the need to find new alternatives [193, 194]. In recent times, decellularized human liver tissue finds application in *ex-vivo* functional maintenance of primary hepatocytes due to its three-dimensional (3D) native liver ECM mimicking architecture which provides excellent bio-mimicking micro-environment for *in vitro* cell growth [195, 196]. However, extensive perfusion processes involved during liver decellularization to remove multiple immuno-regulatory molecules may elicit detrimental immune response while maintaining ECM integrity [197-199]. To overcome these challenges, several attempts have been made by different research groups to create 3D scaffolds from natural or synthetic polymers that mimics native 3D microenvironment to culture hepatocytes [200-204]. Electrospinning is a well-established technique for preparing high throughput nanofibers and to fabricate 3D polymeric scaffolds [205-208], for tissue engineering applications due to the resemblance of nanofibrous 3D matrix with native extracellular matrix [151, 153]. Due to layering of fibers, conventional electrospinning process gives tightly packed fibers resulting in poor cellular infiltration and reduced 3D micro-environment as also discussed in chapter 4. To overcome this issue a liquid assisted collection method, also known as wet electrospinning technique, which was reported by the present lab for improved infiltration of primary human hepatocytes was applied [159, 209]. Chemisorption of collagen-I and fibronectin which are two most abundant macromolecules present in liver matrix [210-212] was used to create a biologically active micro-environment for human liver cells. Collagen-I is found to be a crucial ECM

macromolecular protein for *ex-vivo* maintenance of liver microenvironment, thus enhance liver-specific functions of primary human hepatocytes over the culture period [213-215].

Recent advances in the field of biotechnology have also unfolded the importance of cellular cross-talks among different cells in maintaining biological activities [216-218]. Hepatocytes make up 80% of the liver parenchyma with 20% other non-parenchymal cells, including liver sinusoidal endothelial cells, Kupffer cells, and hepatic stellate cells which perform different role in maintenance of liver-specific functions of primary hepatocytes along with their contribution to inflammatory responses [216, 219]. Specifically, hepatic stellate cells (HSCs), known to secrete hepatocyte growth factor (HGF), stores vitamin A in the form of retinoid droplets and secrete collagen thus possibly involved in vascularization of new tissues which is crucial for survival of functional hepatocytes [220-223]. Although there are multiple studies reported on *in vitro* interactions of hepatocytes with hepatic stellate cells, most of this studies were restricted to culture on tissue-culture plates or spheroid formation or use of cells from rodent origin [224-226], which may be different and less representative to the cellular functions of human liver cells. In addition, researchers also reported a configurational change in primary hepatocytes/HSCs co-culture such as retraction of HSCs from the more of spheroid model might indicate the necessity of optimization of cell microenvironment for proper cellular interactions [225].

To address these challenges, this chapter includes an optimization of primary human hepatocytes (PHHs) to HSCs ratio on a pre-established collagen-I coated electrospun PLGA nanofibers for enhancing viability and functionality of primary human hepatocytes compared to monocultures and gold standard sandwich-cultures.

5.2 Materials and methods

5.2.1 Monocultures of PHHs or HSCs on 3D electrospun PLGA scaffolds

Human hepatic stellate cells were obtained from Samsara Sciences INC, San Diego, CA at passage 0, expanded in complete Dulbecco's Modified Eagle Medium, high glucose (Thermo Fisher Scientific, USA). Utilized HSCs were collected at passage 2 by using Trypan Blue to detect viable cells and seeded on 3D scaffolds. 10^5 cells were seeded on each scaffold ($d = 5\text{mm}$) after sterilization with 70% Ethanol. After 30 minutes of cell attachment, 100 μL of fresh culture medium was added. Cells were then kept in a humidified atmosphere of 5% CO_2 at 37 $^\circ\text{C}$. The media was changed daily. Collagen non-coated PLGA scaffolds were seeded with same number of cells that were used as control. Monoculture of primary human hepatocytes (PHHs) were maintained similarly as it was discussed in the section 4.2.2. in chapter 4.

5.2.2 Co-culture of PHHs and HSCs

In the co-culture, 10^5 PHHs were seeded on each scaffold (5 mm in diameter). After 30 minutes of cell attachment, two different ratios of HSCs were added onto the PLGA-scaffolds previously incubated with PHHs. All the sample types are listed in the table 5.1. Both the cells were incubated for another 30 minutes on the scaffolds before adding another 100 μL fresh HCM medium. All cultures were kept in a humidified atmosphere 37 $^\circ\text{C}$ and 5% CO_2 .

Table 5. 1: Types of PHHs/HSCs cultures

Sample Name	Descriptions	Number of PHHs	Number of HSCs
CP_1:0	C100-coated PLGA scaffold	100 000	0
CP_10:1	C100-coated PLGA scaffold	100 000	10 000
CP_5:1	C100-coated PLGA scaffold	100 000	20 000
CP_0:1	C100-coated PLGA scaffold	0	100 000

P_1:0	Non-coated PLGA scaffold	100 000	0
P_0:1	Non-coated PLGA scaffold	0	100 000
S_1:0	Matrigel Sandwich Culture	100 000	0
S_10:1	Matrigel Sandwich Culture	100 000	10 000
S_5:1	Matrigel Sandwich Culture	100 000	20 000

Collagen-I/Matrigel sandwich culture, known as the gold standard for *in vitro* culture of hepatocytes was used as a control, seeded with the same number of PHHs and HSCs as used for 3D PLGA scaffolds. After seeding cells on collagen-I coated plates, 0.25 mg/mL growth factor-reduced Matrigel Matrix (BD Biosciences) was placed on the cells and incubated at similar culture conditions with 3D PLGA scaffolds.

5.2.3 Cell viability measurement

The viable cells were quantified by using Resazurin assay (Merck Millipore, USA). 10% Resazurin reagent with HCM supplemented to cells and incubated for 4 hours at 37 °C and 5% CO₂. The colorimetric test was carried out at wavelengths of 570 nm and 600 nm using Synergy2 microplate reader (BioTek, USA).

5.2.4 Immunohistochemically analysis

Immunostaining was carried out at predetermined time points. Scaffolds with cells were washed 3 times with PBS before fixing with formalin for 30 minutes. The fixed scaffolds were then embedded in histogel (Thermo Fisher Scientific, USA) before cryo-sectioned for immunohistochemical staining.

5.2.5 Immunocytochemical staining

On day 2 after washing cell-scaffold assembly with PBS (3 times), fixed with 4% formalin solution for 30 minutes at room temperature (RT) followed by permeabilization with 0.1 % Triton-X solution. Then the scaffolds were blocked with

SeaBlock (Sigma Aldrich) for 30 minutes at RT. Cells were immunolabeled by incubating with primary antibodies (chicken anti-albumin, ab16582, 1:100, Abcam and mouse anti-smooth muscle actin, ab7817, 1:100, Abcam) overnight at 4 °C. Then after washing with PBS for 3 times, scaffolds were further incubated with secondary antibodies (goat anti-chicken IgY Alexa Fluor 488, ab 150169, 1:300, Abcam and donkey anti-mouse Alexa Fluor 455, A31570, 1:300, Thermo Fisher Scientific Life Sciences, USA) respectively, in blocking buffer at room temperature for 1 hour. DAPI was used to stain the cell nucleus (1:1000, Invitrogen) by incubating for 5 minutes at room temperature. Nikon A1 Confocal laser microscope system and fluorescence microscope at 10X magnification was used for cell imaging.

5.2.6 Quantitative Real Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

The liver-specific gene expressions of primary human hepatocytes on 3D scaffolds as well as on sandwich cultures were measured by using qRT-PCR. Briefly, TRIzol™ Reagent (Thermo Fisher Scientific Life Sciences, USA) was added in the cultures on predetermined time points to extract the total RNA. Reverse transcription process which was performed by using iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, USA) was used to synthesize the cDNA. Finally, qRT-PCR was carried out by adding SYBR Green PCR reagent (Bio-Rad Laboratories, USA) with cDNA on QuantStudio™ 7 System (Thermo Fisher Scientific Life Sciences, USA). All the data were normalized by using E-cadherin and cyclophilin as housekeeping genes.

5.3 Results

5.3.1 Monocultures on PLGA scaffolds

The viability of PHHs and HSCs monocultures were measured by using Resazurin assay. The presence of collagen-I on 3D fibrous PLGA scaffolds (CP_1:0) enhanced the seeding efficiency of PHHs compared to the cells seeded on unmodified PLGA

scaffolds (P_1:0) on day 2 ($p = 0.0009$) (**Figure 5.1**). However, this effect was absent for HSCs, where no significant difference in cell loading efficiency was observed on day 2 between CP_0:1 and P_0:1 ($p = 0.109$). Cell numbers remain maintained from day 7 to day 14 in all culture types.

Figure 5.1: Surface modification of PLGA nanofibers with collagen-I significantly enhances the seeding efficiency of PHHs monocultures (CP_1:0 vs P_1:0), but there was no significant effect observed on HSCs seeding efficiency (CP_0:1 vs P_0:1). Cell numbers were determined by Resazurin assay ($n = 6$). Error bars indicated standard deviation. * indicates $p < 0.05$.

5.3.2 Co-culture of PHHs and HSCs on PLGA scaffolds

The co-culture of PHHs and HSCs is explored in this section. 10^5 PHHs were seeded on each scaffold and allowed to attach on the scaffold surface for 30 minutes before seeding HSCs at different ratios as mentioned in **Table 5.1**. Viable cells were quantified by Resazurin assay (**Figure 5.2**).

Figure 5.2: PHHs experienced cell number drop on scaffolds in co-culture set up throughout 14-day of culture. Cell numbers as determined by Resazurin assay ($n = 6$). Error bars indicated standard deviation.

On 3D fibrous PLGA scaffolds (CP_1:0, CP_10:1, CP_5:1), cell number was maintained from day 7 to day 14. However, a drop-in cell number was experienced from day 2 to day 7 in all 3D scaffolds due to the loss of non-adherent cells after day 2. However, this phenomenon was absent in sandwich culture due to an overlay of Matrigel on the seeded cells. The similar observation was experienced regarding the drop of cell number from day 2 to day 7 due to the removal of non-adherent cells discussed in the section 4.3.3 under chapter 4.

5.3.3 Expression of liver-specific genes

On day 14, liver specific gene expressions were evaluated by using qRT-PCR (**Figure 5.3**). Albumin and cytochrome P450 genes, known as the basic characteristic genes of hepatocytes, were analyzed for each culture type. A 3 fold and 1.5 fold higher expression of albumin was observed for PHHs when co-cultured with HSCs at a ratio of 10:1 on collagen-I coated 3D scaffolds (CP_10:1) compared to the CP_1:0

monoculture and fresh primary hepatocytes (day 0) respectively. Albumin expression was absent on only HSCs containing scaffolds (CP_0:1).

(A)

(B)

(C)

Figure 5.3: Albumin and CYP2C9 gene expressions (A and B respectively) are up-regulated in co-culture sample CP_10:1 compared to the CP_1:0 monoculture on Day 14, as analyzed through qRT-PCR. Higher expression of CYP3A4 is observed in CP_1:0 monoculture. The gene expressions are normalized to e-cadherin genes which served as PHHs cell number indicator. Error bars indicated standard deviation (n =6).

CYP2C9 and CYP3A4, members of cytochrome P450 family known as metabolism regulatory hepato-specific genes were also studied on day 14. As in Figure 5.3B, in CP_10:1 co- culture group, CYP2C9 gene expression was similar to the fresh primary human hepatocytes. PHHs on CP_10:1 showed ~4-fold higher expression of CYP2C9 compared to both PHHs monoculture (CP_1:0) and CP_5:1 co-culture group. Again, a ~4.5-fold higher expression of CYP2C9 in CP_10:1 was observed compared to the collagen-I/ Matrigel sandwich culture. Taken together, superior CYP2C9 gene expression was maintained by PHHs on CP_10:1 culture group compared to all other sample types, indicating the role of HSCs in maintaining liver specific gene expressions of primary human hepatocytes in addition to the collagen-I coated 3D fibrous microenvironment.

However, CYP3A4 gene expression (**Figure 5.3C**) in the CP_1:0 monoculture was about the same as fresh primary hepatocytes and no further improvement in CYP3A4 gene expression was observed in co-cultures of PHHs with HSCs. This observation may

be indicating the minimal dependence of CYP3A4 gene expression of PHHs on the presence or absence of HSCs *in vitro* on a 3D ECM mimicking collagen-I modified electrospun fibrous scaffolds. However, a ~3-fold higher expression compared to CP_5:1 and ~6-fold higher expression compared to the gold standard sandwich culture of CYP3A4 was recorded for PHHs in CP_10:1 culture groups indicating that a 10 to 1 ratio of PHHs to HSCs might be an optimum combination for *in vitro* co-culture of these two crucial liver cells.

5.3.4 Albumin secretion of PHHs on PLGA scaffolds

The effect of upregulated genes at the translational level was visualized by quantifying albumin secretion by PHHs on 3D PLGA scaffolds (**Figure 5.4**). While being co-cultured, the presence of HSCs enhanced albumin secretion in all co-culture groups. Especially in CP_10:1 culture group, the albumin secretion was highest and reached to 40µg/ day / million cells. Again, albumin production of PHHs on CP_1:0 was higher compared to the S_1:0 at day 2. A decrease in albumin secretion was observed from day 2 to day 14 in all homotypic cultures including CP_1:0 and S_1:0. The albumin secretion was also visualized by immunolabeling on day 2 have shown the similar phenomenon (**Figure 5.5**).

Figure 5.4: Albumin productions are significantly enhanced with the co-culture of PHHs with HSCs compared to monoculture of PHHs (CP_1:0) on collagen-I coated 3D electrospun scaffolds. PHHs on electrospun scaffolds also produced significantly more albumin compared to sandwich cultures. Error bars indicated standard deviation (n = 6).

Figure 5.5: Immunostaining images of Day 2 samples seeded with the same number of PHHs. Sample CP_10:1 displayed more detection of albumin (stained green) among co-culture samples. The presence of HSCs was labeled red with α – SMA antibody. DAPI (blue) is used to stain nuclei. Scale bar = 100 μ m.

5.3.5 Cytochrome P450 Activity of PHHs on PLGA scaffolds

In monocultures and co-cultures, functions of PHHs were further assessed by enzymatic activities of CYP2C9 and CYP3A4 which are two crucial cytochrome P450 genes (**Figure 5.6**) present in human hepatocytes. As observed previously, both CYP3A4 and CYP2C9 gene expressions (**Figure 5.3B and C**) was higher for CP_10:1 and CP_5:1 co-culture groups compared to the sandwich culture of PHHs in S_1:0. This finding indicates that in PHHs-HSCs co-cultures on collagen-I modified 3D electrospun scaffolds, PHHs were able to maintain higher expression of CYP genes compared to the sandwich culture. Similar phenomenon observed in the enzymatic studies of CYP3A4 and CYP2C9 on day 7 and day 14. As observed in **Figure 5.6A**, a slight drop in CYP2C9 and CYP3A4 activity was observed in the co-culture groups from day 7 to

day 14. However, day 14 data showed that a significant improved maintenance of both CYP genes were achieved in CP_10:1 and CP_5:1 co-cultures compared to CP_1:0 and S_1:0 (~8 fold for CYP2C9 and ~3 fold for CYP3A4) monocultures. Thus, presence of HSCs in co-cultures on 3D fibrous scaffolds helped in maintaining better enzymatic activities compared to the monocultures or gold standard sandwich cultures.

Figure 5.6: Both CYP2C9 (A) and CYP3A4 (B) activities are enhanced with the co-culture of PHHs with HSCs compared to the CP_1:0 monoculture of PHHs (CP_1:0) on Day 14. Error bars indicated standard deviation (n = 6). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the CP_1:0 monoculture.

5.4 Discussion

In this study, the importance of co-culture with HSCs to maintain translational and transcriptional properties of PHHs on a pre-established (chapter 4) collagen-I modified native liver ECM mimicking electrospun PLGA scaffolds was demonstrated. In co-cultures, hepato-specific properties of human primary hepatocytes improved significantly compared to the monocultures on 3D PLGA scaffolds and as well as compared to the gold standard collagen-I/Matrigel sandwich cultures. The presence of collagen-I on 3D scaffolds was proven to be crucial for functional maintenance of primary hepatocytes *in vitro* [2]. In this study, the role of collagen-I to improve cell seeding efficiency (**Figure 5.2A**) of 3D PLGA fibrous scaffolds compared to the unmodified PLGA scaffolds was presented. Thus, for further co-culture studies, collagen-I modified 3D electrospun scaffolds were used instead of unmodified 3D nanofibrous scaffolds. The initial seeding efficiency on 3D PLGA fibrous scaffolds was lower compared to the collagen/Matrigel sandwich culture which might be due to the removal of non-adherent cells during change of culture medium. In sandwich culture, removal of non-adherent cell was restricted due to the Matrigel overlay, thus cell number remains nearly constant from day 2 to day 14 in both monocultures and co-cultures (**Figure 5.2A and 5.2B**). Though, cell number decreased from day 2 to day 7 in 3D nanofibrous scaffolds, it remains unaltered from day 7 to day 14, thus indicating the efficiency of the proposed scaffold to maintain viability of primary human hepatocytes *in vitro*. The effect of PHHs/ HSCs co-culture on collagen-I modified 3D electrospun nanofibrous PLGA scaffold was more evident from the enhanced liver specific functions of PHHs evaluated by quantifying the transcriptional level of albumin and cytochrome P450 (CYP2C9 and CYP3A4) genes on day 14 (**Figure 5.3**). In CP_10:1 culture group, the presence of the optimal number of HSCs reinforced transcriptional properties of PHHs, thus maintained higher expression of liver specific genes: 6~fold higher expression of albumin compared to the sandwich culture of PHHs and 3 fold higher compared to the CP_1:0 monoculture group; 8~higher expression of CYP2C9 compared to the PHHs sandwich culture (S_1:0) and 4~fold higher compared to the CP_1:0 culture group; and 6~fold higher expression of CYP3A4 when compared

to the S_1:0. Similarly, by day 14 albumin production was also improved in CP_10:1 co-culture groups by ~1.5 fold compared to CP_5:1, ~4 fold compared to CP_1:0 and ~8 fold compared to S_1:0 (**Figure 5.4**). Taken together, the presence of HSCs with PHHs on 3D fibrous scaffolds reinforced the *ex-vivo* cellular microenvironment which further enhanced functional maintenance of PHHs in the proposed model. This phenomenon further improved the CYP3A4 and CYP2C9 enzymatic activities shown in figure 5.6. Improved 3D configuration provided by electrospun scaffolds itself enhanced the CYP enzymatic activities in both monoculture and co-cultures, however on day 14 the expression were more maintained in the CP_5:1 and CP_10:1 co-cultures compared to both CP_1:0 monoculture group and sandwich cultures. Thus, co-presence of HSCs with primary PHHs can further reinforce cell viability, albumin production by PHHs and regulation of cytochrome P450 enzymatic activities *in vitro*. In this study, PHHs and HSCs were kept in direct contact with each other in same culture conditions just like *in vivo* microenvironment, thus results in better cellular cross-talks by producing different small molecules and growth factor mixtures. Observations from other research groups also strengthen our observations on the co-culture of hepatocytes with hepatic stellate cells on 3D scaffolds. Wei et al. demonstrated the co-culture of rat primary hepatocytes with rat hepatic stellate cells on 3D silk fibroin scaffolds resulted in improved urea and albumin production and CYP enzymatic activities of hepatocytes [227]. Krause et al. also used a co-culture of rat hepatocytes and rat hepatic stellate cells to study glucagon stimulated increase of phosphoenolpyruvate carboxy kinase (PCK) activity in hepatocytes [228]. Compared to the use of rodent cells adopted in the previous publications, this chapter demonstrates the use of human hepatocytes with human hepatic stellate cells for improved maintenance of hepatocytes functionality when co-cultured. Additionally, the importance of 3D nanofibrous microenvironment and ECM in enhancing the viability and functionality of PHHs using collagen-I modified electrospun fibrous scaffolds was highlighted. This study may help in better understanding of culture parameters required to maintain functional human hepatocytes *in vitro* and could also be used to develop a platform for culture of functional liver tissues for various applications such as drug screening.

5.5 Conclusion

In this study, PHHs and HSCs in a series of different initial seeding ratios on collagen-I modified 3D electrospun PLGA scaffolds were co-cultured over 14 days. The results have shown that co-culture of PHH with HSCs on the liver ECM mimicking collagen-I modified 3D electrospun scaffold can improve *in vitro* maintenance of PHHs. Again, a ratio of 10:1 of PHHs to HSCs on collagen-I modified fibrous scaffolds is proven to be optimum for efficient maintenance of translational and transcriptional properties of PHHs over 14-days of culture period. PHHs are the gold standard for studying effect of different drugs and chemicals for *in vitro* studies. Thus, this system deserves further improvisation for application as a potential *in vitro* liver disease model as well as functional hepatocytes associated with transplantation therapies.

Chapter 6

Collagen-I and fibronectin modified three-dimensional electrospun PLGA scaffolds for long-term *in vitro* maintenance of functional hepatocytes

Extracellular matrix (ECM) proteins are important regulators of cellular behaviors in native environment. It has been established that ECM proteins; collagen-I and fibronectin; that are present in liver extracellular matrix, regulate specific functions of primary hepatocytes. This chapter includes the optimization of collagen-I and fibronectin amount for homogeneous modification of 3D electrospun fibrous scaffolds by chemisorption process. Amount of each protein was quantified, and the protein distribution was visualized by using immunostaining technique. Optimum concentration of both the proteins improved in vitro microenvironment for human liver cells. Effect of improved cell microenvironment was studied at the transcriptional and translational level by quantifying liver specific gene expressions, cell viability and albumin secretion at different timepoints. Based on these results, optimum scaffold composition was discussed and concluded in this chapter.

6.1 Introduction

Primary hepatocytes, which represents ~80% of the total liver volume undergoes continuous damage on liver injury [229, 230]. The only possible solution left for end-stage liver diseases is liver transplantation. However, due to shortage of donor organs, many research labs focusing on tissue engineering approaches to fabricate artificial grafts with functional hepatocytes [231]. Three-dimensional (3D) cultures with copious cell-cell and cell-matrix interactions have been introduced as a better *in vitro* model for mimicking complex *in vivo* cellular microenvironment [232, 233]. Hepatocytes rapidly lose their liver specific functions in 2D cultures, whereas in 3D cultures, hepatocytes were able to maintain their functionality and viability over the culture period [234, 235]. Thus, 3D cultures with functional liver cells were able to produce better insights of the *in vivo* counterpart. Thus it could be useful to evaluate effect of different fibrosis causing chemicals and to find potential cures for liver diseases [236-239].

Electrospinning has been emerged as a simple, versatile and cost-effective technique to prepare fibrous scaffolds for tissue engineering applications [240-243]. Liver extracellular matrix, which have a random fibrous structure can be closely mimicked by the 3D random fibrous scaffolds produced by electrospinning process [244-246]. Wet electrospun 3D fibrous scaffolds modified with ECM proteins have shown improved pore size and porosity as it was discussed previously in chapter 4 and 5 [247]. ECM proteins are important regulators of cellular functions [139, 248, 249]. Collagens and fibronectins are crucial macromolecular proteins present in liver ECM, maintains cellular cross-talks, functions and cell phenotype [15, 250]. Studies revealed differential effects of collagen and fibronectin on cell attachment, cell-cell interactions and functional maintenance of hepatocytes *in vitro* [140, 251-254]. The effect of collagen-I or fibronectin modification on viability, functionality and cellular interaction of primary human hepatocytes cultured on 3D electrospun PLGA scaffolds was discussed in chapter 4 [2]. Wang et al. reported that PEG-ICC scaffolds modified with fibronectin and collagen can differentially regulate the gene expression patterns and liver specific functions of human hepatocytes. They show that ICC scaffolds modified with

fibronectin maintain liver specific functions such as albumin and cytochrome P450 gene expressions of Huh-7.5 cells and cell-signaling related genes such as occludin, cludin and integrin beta1 significantly higher compared to the scaffolds modified with collagen-I [140]. These studies demonstrate the importance of individual collagen-I and fibronectin for *in vitro* culture of hepatocytes. However, the combined effect of these two important ECM proteins on maintenance of hepatic functions of human hepatocytes remain to be explored.

This chapter includes the effect of the co-presence of collagen-I and fibronectin on the maintenance of liver specific functions of human hepatocytes on 3D electrospun nanofibrous scaffolds. The surface of electrospun sub-micron fibers was modified with both collagen-I and fibronectin and the optimum ratio of these two ECM proteins was optimized to achieve efficient protein distribution on the scaffold surface. Huh-7.5 cells, which is a liver carcinoma cell line and highly permissive to hepatitis C virus (HCV) infection was used as a model cell to study the effect of differential scaffold modification on the viability and functionality of human hepatocytes. Our study reveals the synergistic effect that collagen-I and fibronectin scaffold modifications have on cell proliferation and gene expressions of Huh-7.5 cells. In comparison to unmodified PLGA (poly(lactic-co-glycolic acid) nanofibrous scaffolds and single protein modified scaffolds, both collagen-I and fibronectin modified electrospun fibrous scaffolds shown improved maintenance of hepatic gene expressions and viability over a 28 days of culture period.

6.2 Materials and methods

6.2.1 Fabrication and surface modification of electrospun scaffolds

The detailed description of the preparation of wet electrospun 3D scaffolds has been described in a previous publication [255] . Briefly, poly(lactic-co-glycolic acid) 80:20 (Evonik Industries, Essen, Germany) was dissolved in 7:3 chloroform to dimethylformamide (DMF) (Fisher Scientific, Waltham, MA, USA) and electrospun

using NANON-01A instrument (MECC, Fukuoka, Japan) over a fiber collection bath. The fibers were collected in a metal mold (10 mm diameter) and freeze-dried to obtain 3D electrospun PLGA fibrous scaffolds. Fiber surface was modified with rat-tail Collagen-I and plasma fibronectin (Life Technologies, Carlsbad, California, USA) at five different ratios by chemisorption process. All scaffold types are listed in **Table 6.1**.

Table 6.1: Type of scaffolds prepared by electrospinning followed by chemisorption process.

Scaffold Type	Collagen added for surface modification per Scaffold (μg)	Fibronectin added for surface modification per Scaffold (μg)	Total protein added (μg)
C:F_0:0	0	0	0
C:F_1:0	100	0	100
C:F_0:1	0	50	50
C:F_1:1	50	50	100
C:F_3:1	75	25	100
C:F_9:1	90	10	100

The surface modification was performed by surface hydrolysis of the PLGA fibers with 0.01 (N) NaOH (Sigma Aldrich, St. Louis, MO, USA) followed by activation with 40 mM EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide) and 80 mM NHS (N-hydroxysuccinamide) in 50 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH = 6). After washing with DI water, the scaffolds were treated with collagen-I and/or fibronectin solution of different concentration overnight at 4 °C under continuous shaking and freeze-dried to stabilize the shape. For multiple protein modification, both Collagen-I and fibronectin added to the reaction medium simultaneously and kept at 4 °C overnight under continuous shaking to improve protein distribution on the scaffold surface.

6.2.2 Characterization of ECM modified fibrous scaffolds

6.2.2.1 Western Blot

Western blot was used to quantify the individual protein content of the ECM modified scaffolds. The leftover protein solution after modification of fibrous scaffolds was

collected for western blot analysis. Protein-modified scaffolds were washed three-times with DI water and washings were collected to calculate the amount of total protein that did not bind to the scaffolds. Diluted protein solutions were freeze-dried and re-dissolved in known amount of PBS for western blot analysis. The samples were boiled with NuPAGE™ Sample Reducing Agent and NuPAGE LDS Sample Buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States) for 5 minutes and loaded (20 µl) into the wells of 7% NuPAGE Tris-Acetate mini gels (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Electrophoresis was applied at a voltage of 150 V. On completion of electrophoresis, the proteins were trans-blotted to a PVDF (Polyvinylidene fluoride) membrane at 125 mA for 9 minutes and blocked with 3% bovine serum albumin (BSA) in 0.1% PBST (Phosphate buffered saline with 0.1% Tween-20) for 1 h at room temperature. The primary antibodies (Rabbit polyclonal to Collagen I (ab21287), Mouse monoclonal [HFN 7.1] to Fibronectin (ab80923; Abcam, Cambridge, United Kingdom) were diluted at a 1:1000 ratio in 0.1% PBST with 3% BSA (Bovine serum albumin) and incubated with the membrane at 4°C overnight. After incubation, the membrane was washed with 0.1% PBST three time (5 minutes each) and incubated in secondary antibodies (Goat Anti-Rabbit IgG H&L (HRP) (ab6721); Goat Anti-Mouse IgG H&L (HRP) (ab6789; Abcam, Cambridge, United Kingdom)) diluted with 3% BSA in 0.1% PBST at a ratio of 1:10000. The immunoreactive bands on the immunoblot were detected by using a luminol based signal enhancer (chemiluminescent substrate; Thermo Fisher Scientific). The intensity of the bands was measured with imageJ software and normalized to standard bands composed of known quantities of collagen-I and fibronectin. Triplicates were performed for each sample type in all western blot experiments.

6.2.2.2 Immunofluorescence staining

Immunofluorescence staining was used to visualize the distribution of the collagen-I and fibronectin on the fibrous scaffold. The scaffolds were washed with PBS and fixed in 4% paraformaldehyde (PFA) for 5 minutes followed by blocking with 3% BSA in 0.1% PBST. The protein fixed scaffolds were washed with PBS thrice and incubated in

respective primary antibodies (1:1000 dilution in blocking solution) for collagen-I (Rabbit polyclonal to Collagen I (ab21287; Abcam, Cambridge, United Kingdom) and fibronectin (Goat Anti-Mouse IgG H&L (HRP) (ab6789; Abcam, Cambridge, United Kingdom) at 4°C overnight. The scaffolds were washed thoroughly with PBS and incubated with the secondary antibodies after recommended dilution in 0.1% PBST containing 3% BSA for 2 hrs. at room temperature. Goat anti-rabbit IgG H&L (1:500 dilution in blocking solution, Alexa Fluor® 568) (ab175471) (Red) was used to stain collagen-I and goat anti-mouse IgG H&L (1:500 dilution in blocking solution, Alexa Fluor® 488) (ab150117) (Green) was used for fibronectin. The fluorescence images of the stained samples were captured on the confocal microscope (Carl Zeiss LSA710).

6.2.3 Culture of Huh-7.5 cells on 3D electrospun scaffolds

Huh-7.5 cells (Apath, NY) are a hepatocellular carcinoma cell line. Huh-7.5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% antibiotic (100 U/ml penicillin, 100 µg/ml streptomycin, Gibco) at 37°C in the presence of 5% carbon dioxide (CO₂). Scaffolds were sterilized under UV light for 30 minutes on each side. Both the protein modified, and unmodified scaffolds were kept immersed in the culture media over night for allowing penetration of the media inside the scaffolds. 0.1×10^6 cells were seeded on each scaffold with a working volume of 20 µl. After incubating overnight, the scaffolds were transferred to a new 12 well plate with 1 ml media in each well and maintained in 5% CO₂ at 37°C. Culture media was changed every 2 days.

6.2.4 Cell seeding efficiency and proliferation

The cell seeding efficiency and proliferation of the Huh-7.5 cells on the PLGA fibrous scaffolds were measured by using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, MD). Seeding efficiency of the Huh-7.5 cells was measured after overnight incubation of the cells on the PLGA scaffolds. Cells were incubated in complete culture medium containing 10% CCK-8 solution for 2 hrs. at 37°C. The

supernatant (100µl in each well) was transferred to a 96-well plate and the absorbance was measured at 450 nm in a SpectraMax M2 multi-mode microplate reader (Molecular Devices, LLC., San Jose, USA).

Cell proliferation was measured at day 1, 7, 14, 21 and 28 by using CCK-8 assay. A standard curve for the CCK-8 assay was made by performing the assay on standard wells with a known number of plated cells. The absorbance values of control wells were plotted against the known cell numbers and a linear regression was performed. This linear fit equation was used to relate absorbance values obtained at each time point to the total number of cells present in each scaffold.

6.2.5 Live-Dead assay

A live-dead viability/cytotoxicity kit (Life Technologies, California, United States) was used to visualize the cell viability on day 2 and 28. Calcein AM (4 µM) and ethidium holodimer-1 (EthD-1, 8 µM) were mixed with complete media and incubated with cells at 37°C for 30 mins. Hoechst 33342 solution (Thermofisher scientific, Massachusetts, United States) was used to stain the nucleus of the cells. The live and dead cells were stained green and red, respectively, and nuclei was stained blue. The images were captured using an Olympus U-RFL-T fluorescent microscope (Olympus Corporation, Tokyo, Japan).

6.3 Results

6.3.1 Surface modification and characterization of 3D electrospun PLGA scaffolds

Three different ratios of collagen I to fibronectin (C:F) with a total concentration of 100 µg/ml were used for surface modification of the fibrous scaffolds (**Table 6.1**). The scaffolds after modification were observed under SEM as shown in Figure 6.1. Surprisingly, the different proteins as well as the different ratios gave rise to different adsorption patterns. When comparing single protein, fibronectin was shown to be

uniformly distributed on the scaffold fibers, whereas significant aggregation of collagen was observed. When both proteins were used but at varying ratios, the difference was not as apparent (the lower row).

These scaffolds were further immuno-stained to have a clearer picture of the surface modification with macromolecular proteins. As can be seen in **Figure 6.2**, the Z-stack immunostaining images of single protein modification (upper row) corroborate well with the SEM images where fibronectin was homogeneously distributed as opposed to the aggregation of collagen on the scaffolds.

For scaffolds modified with both collagen-I and fibronectin proteins, Z-stack images of immunostaining revealed interesting patterns across the different ratios of proteins used (**Figure 6.2**, lower row). Among C:F_1:1, C:F_3:1 and C:F_9:1 samples, a uniform distribution of both collagen-I and fibronectin was achieved in C:F_3:1 scaffolds. In C:F_1:1 scaffolds, immunostaining showed aggregation of collagen-I and fibronectin, whereas in C:F_9:1 scaffolds, the protein distribution is not uniform across the scaffold. The SEM and Z-stack images of the C:F_3:1 scaffolds indicate that both collagen-I and fibronectin were present all over the scaffold with a more homogeneous distribution compared to both C:F_1:1 and C:F_9:1 scaffolds.

Figure 6.1: SEM images of unmodified (C:F_0:0) and ECM protein modified scaffolds (C:F_1:0, C:F_0:1, C:F_1:1, C:F_3:1 and C:F_9:1). Scale bar = 10 μ m.

Figure 6.2: Immunostaining of Collagen-I and fibronectin in the ECM modified electrospun PLGA scaffold. Collagen-I was stained with Alexa Fluor® 568 (Red) and Fibronectin was stained with Alexa Fluor® 488 (Green)

FTIR analysis confirms the presence of proteins on each type of surface modified scaffold (**Figure 6.3**). The presence of amide-I, amide-II and amine-III bonds were confirmed from the peaks obtained at 1640 cm^{-1} due to the stretching vibrations of peptide C=O groups, at 1566 cm^{-1} originating from the bending of N-H bond and at 1267 cm^{-1} due to stretching of C-N group. Peaks related to amide-I, amide-II and amide-III were absent in the spectra of unmodified PLGA scaffolds (C:F_0:0) as expected. N-H stretching frequency due to the amine group ($-\text{NH}_2$) results in a broad band at $3200\text{--}3500\text{ cm}^{-1}$ for the scaffolds modified with ECM proteins. The total concentration of the proteins immobilized on each type of scaffold was evaluated using a BCA assay, while the total amount of collagen 1 and fibronectin present on modified scaffold surfaces was measured using western blot analysis (**Table 6.2**). The total amount of protein used to modify C:F_1:0, C:F_1:1, C:F_3:1 and C:F_9:1 scaffolds was statistically indifferent, but significantly higher protein content was achieved in C:F_3:1 scaffold upon modification by chemisorption method.

Figure 6.3: Characterization of protein modified scaffolds. FTIR spectra of unmodified and protein modified scaffolds. The broad peaks at $3200\text{--}3500\text{ cm}^{-1}$ corresponds to the N-H

stretching frequency of primary amine. Peaks at 1640 cm⁻¹, 1566 cm⁻¹ and 1267 cm⁻¹ correspond to the stretching /bending of amide-I, amide-II and amide-III groups, respectively.

The difference in total protein content found from the BCA assay (n=3) and western blot analysis (n=3) was not statistically significant. It was also observed that the ratio of collagen-I to fibronectin for C:F_1:1 and C:F_3:1 was approximately 1:1 and 3:1, however for C:F_9:1 the ratio was approximately 14:1. Thus, the theoretical ratio of collagen-I to fibronectin was maintained in C:F_1:1 and C:F_3:1, but it is not maintained in C:F_9:1 after surface modification. Again, C:F_3:1 scaffolds found to have significantly higher content of total protein compared to C:F_1:1 and C:F_9:1.

Table 6.2: Quantification of collagen and/or fibronectin in the protein modified scaffolds

Scaffold type	Total ECM protein (µg/mg of PLGA) Added for modification	Immobilized collagen (µg/mg of PLGA) quantified by Western Blot	Immobilized fibronectin (µg/mg of PLGA) quantified by Western Blot	Total ECM protein (µg/mg of PLGA) quantified by Western Blot	Total ECM protein (µg/mg of PLGA) quantified by BCA assay	<i>p</i> value (comparison between the data obtain by western blot and BCA assay)
C:F_1:0	8.9±0.4	-	-	4.7±0.3	4.4±0.3	0.202
C:F_0:1	4.8±0.5	-	-	2.5±0.6	2.4±0.5	0.064
C:F_1:1	9.6±4.3	2.1±0.5	2.4±0.5	4.5±0.2	5.7±1.3	0.097
C:F_3:1	8.5±0.4	6.1±0.1	2.0±0.5	8.0±0.5	8.4±0.4	0.396
C:F_9:1	7.9±1.1	5.6±1.2	0.4±0.2	6.0±1.3	4.9±1.0	0.383

6.3.2 Culture of Huh-7.5 cells on ECM modified 3D electrospun scaffolds

The viability, gene expression, and hepatic functions of Huh-7.5 cells cultured on protein-modified scaffolds were quantified over 28 days. First, the effect of protein modification on the cell seeding efficiency was indicated by measuring cell viability on the 3D electrospun scaffolds after 24 hours culture. **Figure 6.4A** shows that the protein modification of the scaffolds improved cell seeding efficiency significantly compared

to the unmodified 3D PLGA scaffolds ($p < 0.001$). The cell seeding efficiency of the fibronectin modified scaffolds i.e. C:F_0:1 was significantly higher compared to C:F_1:0 scaffolds ($p < 0.05$), indicating that Huh-7.5 cells did not adhere to collagen-I as much as to fibronectin during initial cell seeding. No difference in cell seeding efficiency was observed for the scaffolds containing both collagen-I and fibronectin to that of C:F_0:1 scaffolds. **Figure 6.4B** shows that cell proliferation for all scaffold types were similar until day 14. 4-fold increase of cell number was observed for the unmodified (C:F_0:0) and collagen-I/fibronectin modified scaffolds after 7 days of culture. The cell numbers remain unaltered for unmodified (C:F_0:0) and single protein modified (C:F_1:0 and C:F_0:1) culture groups from day 7 to day 28. A gradual increase in cell number was observed from day 7 to day 28 for the cultures on the scaffolds modified with both collagen-I and fibronectin, especially on C:F_3:1 and C:F_9:1 group. On C:F_1:1 scaffolds, no further increase in cell number was experienced from day 21 to 28. At the end of 28 days of culture, the cell number increased by 6-fold, 10-fold and 8-fold for C:F_1:1, C:F_3:1 and C:F_9:1 culture groups respectively.

Figure 6.4: Cell seeding efficiency and cell proliferation assay. (A) 1×10^5 cells were seeded on each scaffold and cell seeding efficiency measured as the viability of cells after overnight

incubation at Day 1. (B) Cell proliferation was measured on Day1, 7, 14, 21 and 28 by using CCK8 assay (n=3). (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared to the unmodified PLGA scaffolds (C:F_0:0))

Live/dead images of the Huh-7.5 cells cultured on the electrospun 3D scaffolds were captured on day 2 and day 28 (**Figure 6.5**). The cell density was observed to increase from day 2 to day 28 in all scaffold types. Most of the cells were alive at day 28. However, higher number of dead cells were visible on the C:F_0:0 scaffolds on day 28 when compared to cells cultured on protein modified scaffolds.

Figure 6.5: Live/dead staining images of Huh-7.5 cells on 3D electrospun PLGA scaffolds. The images taken on day 2 and day 28 showing that the cell number was increased significantly

for all scaffold types over the culture period. Calcein-AM was used to stain the live cells with green-fluorescence and EthD-1 with-red fluorescence stained the dead cells. Scale bar = 100 μm .

6.3.3 Liver specific gene expressions

The liver specific gene expression of each culture groups was quantified using qRT-PCR at day 2, 7, 14, 21 and 28. The data was presented as a fold change normalized to the C:F_0:0 culture group at the specific time points (**Figure 6.6**). Albumin and cytochrome P450 genes are the most important liver specific genes defining the characteristics of hepatocytes. One of the prime functions of hepatocyte is albumin production, therefore studying albumin expression is one of the standard characterizations to indicate functionality of liver cells. Generally, the albumin gene expression was significantly higher for the cells cultured on protein modified scaffolds compared to the cells cultured on unmodified scaffolds (**Figure 6.6A**) across the time points studied. The trend is similar when comparing single protein to two proteins modification, where the latter generally showed higher albumin gene expression. Specifically remarkable outcome is elicited by the C:F_3:1 scaffolds where the albumin gene expression increased by 12.5 folds and about 7.9 and 10.9 folds when compared to unmodified, collagen-I modified and fibronectin modified scaffolds respectively, by day 28 of culture.

(C)

Figure 6.6: Gene expressions of (A) Albumin (ALB), (B) CYP3A4 and (C) CYP3A7 in Huh-7.5 cells on the nanofibrous scaffolds. qPCR analysis was performed to quantify the gene expression of Huh-7.5 cells on day 2, 7, 14, 21 and 28. Data is presented as the fold change over the control group (C:F_0:0) (N=3, mean $\pm$ SD). The significance of fold change over the cells cultured on C:F_1:0 is expressed as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Cytochrome P450 genes play important role in oxidation of host xenobiotic components [256]. CYP3A4 and CYP3A7 genes, members of cytochrome P450 genes are important markers of functional hepatocytes and regulators of hepatocytes metabolic activity [140]. The effect of protein modification on these two genes was studied over 28 days of culture and shown in **Figure 6.6B** and 6.6C. Although the general effect was not as apparent as in albumin gene expression, the result of C:F 3:1 scaffold remained exceptional. The CYP3A4 and CYP3A7 genes expression were significantly higher in cells cultured on C:F 3:1 scaffolds as compared to that on unmodified as well as ECM modified scaffolds throughout the timeframe studied. At day 28, the gene expressions had increased by 7.3 and 4.5 folds as compared to control for CYP3A4 and CYP3A7 respectively. These gene expression studies indicate the importance of the presence of both ECM proteins at optimum level in the cell microenvironment.

6.3. 4 Albumin secretion from Huh-7.5 cells cultured on 3D electrospun PLGA scaffolds

Albumin production is one of the most fundamental characteristics of functional hepatocytes. Therefore, quantification of albumin secretion is an important technique to measure synthetic functions of hepatocytes *in vitro*. Huh-7.5 cells was examined for albumin secretion over 14 days of culture (**Figure 6.7**).

Figure 6.7: Albumin secretion by Huh-7.5 cells on the 3D electrospun PLGA scaffolds. The data was collected on day 2, day 7 and day 14 (N=3, mean±SD). The significance of albumin synthesis of Huh-7.5 cell cultured on protein modified scaffolds compared to the cells cultured on C:F_0:0 is expressed as: # $p<0.05$; ## $p<0.01$; ### $p<0.001$; compared to cells cultured on C:F_1:0 is expressed as: * $p<0.05$.

Initially at day 2, all cultures on protein modified scaffolds shown significantly higher secretion of albumin compared to the control: (C:F_0:0 = 0.6 ± 0.1 $\mu\text{g}/10^6\text{cells/day}$; C:F_1:0 = 1.3 ± 0.3 $\mu\text{g}/10^6\text{cells/day}$; C:F_0:1 = 1.4 ± 0.3 $\mu\text{g}/10^6\text{cells/day}$; C:F_1:1 =

1.2±0.3 µg/10⁶cells/day ; C:F_3:1 = 1.1±0.2 µg/10⁶cells/day; C:F_9:1 = 0.9±0.1 µg/10⁶cells/day). After 7 days of culture, the albumin production by Huh-7.5 cells on all 3D scaffolds become statistically indifferent. However, after 14 days of culture, the cells cultured on C:F_3:1 (C:F_3:1 = 6.7±1.8 µg/10⁶cells/day) scaffolds shown ~4 fold increase in albumin secretion over C:F_0:0 (C:F_0:0 = 1.8±0.6 µg/10⁶cells/day) culture group, ~3 fold compared to C:F_1:0 (C:F_1:0 = 2.5±0.5 µg/10⁶cells/day) culture group and ~2 fold compared to C:F_0:1 (C:F_0:1 = 3.3±0.1 µg/10⁶cells/day) culture group indicating the importance of ECM in the cell microenvironment to maintain liver specific functions over prolonged culture period. All multi-protein modified culture groups showed significantly higher (p<0.05) production of albumin after 14 days of culture over the control (C:F_0:0 = 1.8±0.6 µg/10⁶cells/day; C:F_1:1 = 3.7±0.6 µg/10⁶cells/day; C:F_3:1 = 6.7±1.8 µg/10⁶cells/day; and C:F_9:1 = 3.1 ± 0.2 µg/10⁶cells/day). However, C:F_3:1 culture groups manifest highest albumin production compared to both C:F_1:1 (~1.8 fold) and C:F_9:1 (~2.1 fold) culture groups.

6.4 Discussion

ECM proteins are essential in creating favorable microenvironment for cells to regulate their functions and behavior *in vitro*. Both collagen-I and fibronectin are major ECM proteins present in human liver and regulate functions of liver cells both *in vivo* and *in vitro*. The effect of these proteins on hepatocyte functions have been investigated on different 2D platforms by many research groups [257-259]. However, due to the absence of 3D environment, hepatocytes de-differentiate rapidly and lose their functions in 2D culture systems[146, 260]. In the previous study, it was found that the primary human hepatocytes cultured on 2D PLGA electrospun fiber mat lose their viability as well as liver specific functions at very early time points [261]. In this past work, collagen-I modified PLGA electrospun fibrous scaffolds maintained higher liver specific gene expressions of the human primary hepatocytes over 14 days of culture when compared to the gold standard sandwich culture which was discussed in chapter 4 [2]. It is well documented in previous publications that cells can secrete their own

ECM proteins to remodel their surroundings [262]; however, the existing ECM proteins can initially stimulate this process [140]. In the present study, it was observed that scaffolds modified with both collagen-I and fibronectin improved cell microenvironment of the Huh-7.5 cells on 3D electrospun PLGA scaffolds. Huh-7.5 cells were cultured for 28 days on three different types of scaffolds: (A) unmodified 3D PLGA nanofibrous scaffolds (C:F_0:0) as a control; (B) either collagen-I modified (C:F_1:0) or fibronectin modified (C:F_0:1) scaffolds and (C) scaffolds modified with both protein types i.e. collagen-I and fibronectin at three different ratios (C:F_1:1, C:F_3:1 and C:F_9:1). The protein amount on each scaffold types quantified by BCA and western blot analysis revealed that a collagen-I: fibronectin mass ratio of 3:1 resulted in the highest absolute amount of total protein chemisorbed on PLGA scaffolds ($p < 0.5$) compared to C:F_1:0, C:F_1:1 and C:F_9:1 scaffolds. The final protein amount chemisorbed onto each scaffold was lower than the initial amount of protein introduced indicating that the protein components involved in the chemisorption reaction were in excess. While the initial ratio of collagen and fibronectin was preserved after the chemisorption reaction for the C:F_1:1 and C:F_3:1 scaffolds, this phenomenon was absent in C:F_9:1 scaffolds which may be attributed to the fact that the size of collagen-I (~300nm) is much larger than the size of fibronectin (~120nm) molecules [263, 264]. Thus, the higher concentration of collagen-I molecules may limit interactions between fibronectin molecules and the PLGA fiber surface. The immunofluorescent Z-stack images of protein-modified 3D scaffolds (**Figure 6.2**) shows that the collagen-I: fibronectin ratio plays an important role in the distribution of protein on the scaffold surface. Thus, it also regulates the efficiency of the chemisorption process to modify the fiber surface for each scaffold type. Scaffolds modified with collagen-I (C:F_1:0) have aggregation of the protein molecules at different locations which may be due to the better interaction of collagen-collagen molecules relative to collagen-substrate (PLGA fiber) interaction. Again, for fibronectin-modified scaffolds, uniform distribution of the protein throughout the fiber surface was achieved probably due to the smaller size of fibronectin molecules as well as higher affinity of fibronectin towards the substrate. In case of scaffolds containing both the collagen-I and fibronectin, it was observed that presence of fibronectin could

further influence the distribution of collagen on scaffold surface. Equal amounts of fibronectin and collagen-I (C:F_1:1) reduce the aggregation of the collagen when compared to the scaffolds modified with only collagen-I molecules. However, homogenous protein distribution was still not achievable. In C:F_9:1 scaffolds, the distribution of protein was non uniform which was probably due to the low concentration of fibronectin available. This deficiency prevented a uniform and complete coverage of fibronectin on the scaffolds. C:F_3:1 scaffolds have a uniform distribution of the two ECM proteins throughout the scaffold surface making this ratio optimal for modifying electrospun scaffolds by the chemisorption process. The total protein concentration in C:F_3:1 scaffolds was also significantly higher compared to the other two types of multiprotein modified scaffolds i.e. C:F_1:1 and C:F_9:1, even though the initial concentration of total protein added to modify all these scaffolds were similar.

The seeding efficiency of Huh-7.5 cells cultured on these 3D electrospun scaffolds was higher in fibronectin modified fibrous scaffolds (C:F_0:1) compared to blank (C:F_0:0) and collagen-I modified scaffolds (C:F_1:0) (**Figure 6.4A**) indicating the importance of the presence of fibronectin for cell attachment on the scaffolds. No significant difference in cell proliferation was observed between unmodified and single protein modified culture groups until day 28 (**Figure 6.4B**) indicating that presence of collagen-I or fibronectin may have an effect on cell attachment, but have very less effect on cell proliferation at the later time points (day 7, 14 and 28). In multiprotein modified culture groups, no difference in cells proliferation was observed with the other culture types until day 14. However, the cell number increased significantly for C:F_3:1 and C:F_9:1 scaffolds at day 21 and 28. The total protein content of C:F_1:0 and C:F_9:1 scaffolds is statistically similar, though in the latter, presence of fibronectin on the scaffold surface shows improved proliferation of Huh-7.5 cells specially at the later time points (after day 14). Therefore, fibronectin provides a synergistic effect with collagen to maintain the viability of Huh-7.5 cells on 3D electrospun fibrous scaffolds. In C:F_1:1 culture groups, presence of fibronectin was not able to effect the fold change of Huh-7.5 cells after day 21. This may be due to the significantly lower content of collagen-I

on C:F_1:1 scaffolds compared to C:F_3:1 and C:F_9:1 scaffolds. C:F_3:1 scaffolds, which contains maximum amount of total protein showed improved cell proliferation and highest number of cells at the end of 28 days compared to both C:F_1:1 and C:F_9:1 culture groups ($p < 0.05$). Thus, the amount of collagen-I and fibronectin, as well as the total amount of both the proteins present on each scaffold types are important regulators of cellular functions.

qRT-PCR analysis (**Figure 6.6**) conducted at different time points indicated that all protein-modified scaffolds were able to maintain significantly higher expression of albumin, CYP3A4 and CYP3A7 at all times points till 28 days compared to unmodified scaffolds. However, after day 7, it was observed that the culture groups containing both collagen-I and fibronectin modified scaffolds shown significantly higher albumin gene expression when compared with culture groups containing only collagen-I modified scaffolds (C:F_1:0) stating the importance of the presence of both the proteins to create preferable cellular microenvironment. In C:F_3:1 scaffolds, albumin gene expression was the highest at the end of 28 days indicating that the total protein content as well as the homogeneous distribution of the proteins on the scaffold surface improves albumin gene expression for *in vitro* cultures. Again, along with C:F_3:1, both C:F_1:1 and C:F_9:1 were able to maintain better expression of albumin gene compared to the unmodified and single protein modified scaffolds. This observation indicating the importance of the presence of fibronectin in the cell microenvironment which regulates cellular functions both in *in vitro* and *in vivo* culture of the liver cells. For CYP3A4 and CYP3A7 gene expression, C:F_3:1 scaffolds were able to maintain significantly higher expression compared to all other conditions which may be attributed to the higher amount of total protein content and better distribution of the proteins on these scaffolds. The albumin secretion by Huh-7.5 cells measured on day 14 also complemented the data obtain by qPCR assay. It was found that at the end of day 14, C:F_3:1 cultures shown better secretion of albumin compared to the C:F_1:0 cultures and the cultures maintained on unmodified PLGA scaffolds. Thus, the optimized protein concentrations induced albumin secretion for long-term culture of Huh-7.5 cells.

Both collagen-I and fibronectin play distinct roles in maintenance of cellular functions in liver. This study demonstrates that including both proteins on the 3D fibrous scaffold can synergistically improve the viability and functionality of Huh-7.5 cells in a long-term culture system. The optimum amount of each protein type in chemisorption process can have a significant role on the protein distribution and total protein modification on 3D fibrous scaffolds. The functionalization of fibrous scaffolds with collagen-I and fibronectin found to have effect on cell proliferation and maintenance of hepatic gene expression, especially at later time points. These results show that cellular microenvironment is very crucial in maintaining long-term *in vitro* culture of liver cells. These systems can have a great impact for future studies with human primary hepatocytes and together with the 3D porous electrospun scaffold, could provide a better platform, which can closely mimic the *in vivo* microenvironment of human liver.

6.5 Conclusion

Collagen-I and fibronectin are essential ECM components in the *in vivo* microenvironment of liver cells. In this work, we are interested to investigate the synergistic effect of both proteins on 3D scaffolds in supporting, particularly, the functional properties of liver cells. The effect of individual protein has been widely studied and especially so in 2D scaffolds.

Here it was established that the optimal ratio of collagen-I to fibronectin is crucial in promoting efficient chemisorption of the proteins on our 3D highly porous and fibrous scaffold. The fibronectin presence at the right concentration is also essential to facilitate the homogenous distribution of collagen in the scaffold. Both higher chemisorption and better homogeneity of the proteins led to better support of viability and functional properties of the Huh 7.5 cells over long-term culture of 28 days.

Specifically, the ratio of C:F 3:1 was found to be the most efficient in the support of liver cell functionality. The gene expressions of albumin, CYP3A4 and CYP3A7 increased by ~12.5, 7 and 4.5 folds respectively as compared to unmodified scaffolds.

Compared to only collagen or only fibronectin modified scaffolds the albumin gene expression of cells cultured on C: F_3:1 scaffold increased between ~8 to 10-fold whereas CYP3A4 gene expression improved between ~5 to 7-fold and CYP3A7 gene expression improved by ~4 to 4.5-fold after a long culture period of 28 days. Albumin production by Huh-7.5 cells was also improved by ~4-fold compared to unmodified PLGA scaffolds, ~3-fold compared to collagen-I modified culture groups and ~2-fold compared to fibronectin modified culture groups.

In conclusion, these results point to the importance of multi-protein and optimum ratio of these proteins in designing an effective scaffold for *in vitro* liver cell culture. This platform could set the base for future culture of primary human hepatocytes in liver tissue engineering which would find potential in drug screening and translational applications.

Chapter 7

3D Electrospun nanofibrous scaffolds with functional liver cells to study alcoholic liver fibrosis *in vitro*

This chapter incorporates the fabrication of an alcoholic liver disease model (ALD model) by using ECM modified electrospun 3D fibrous scaffolds. A variety of characterization techniques are discussed in this chapter to optimize the ideal scaffold to fabricate an in vitro ALD model. A co-culture of primary human hepatocytes with LX-2 cells was established on the 3D electrospun nanofibrous scaffolds. The gold standard sandwich culture was used as a control. Liver-specific gene expressions i.e. albumin and cytochrome P450 gene expressions were evaluated at different alcohol concentrations along with alcohol untreated culture groups. Alcohol treatment, as well as the concentration of alcohol in the culture medium, were found to affect the phenotype and functions of both PHHs and LX-2 cells. Favorable cell-microenvironment was found to regulate liver-specific functional characteristics in the proposed in vitro model. These findings pave a promising path to create an artificial scaffold, not only to support functional human primary hepatocytes for liver tissue engineering but also to examine the effect of different drugs or chemicals in progression or inhibition of liver fibrosis.

7.1 Introduction

One of the prevalent causes of hepatic fibrosis in industrialized countries is long term alcohol abuse, which responsible for ~10% of all the deaths caused by disease in the world [265, 266]. Alcohol injury causes steatosis which gradually progresses to fibrosis and cirrhosis, if remain untreated, results in hepatocellular carcinoma [267]. Hepatic stellate cells (HSCs), a non-parenchymal cell of the liver stores vitamin A in the form of retinoid droplets. Repeated alcohol injury initiates activation of the hepatic stellate cells identified as the central event in hepatic fibrosis. Once activated, HSCs losses its quiescent phenotype and trans-differentiate to myofibroblasts. Activated hepatic stellate cells produce excessive extracellular matrix and impair healthy architecture of the liver [268, 269]. Alcoholic liver fibrosis gradually progresses to an irreversible stage, clinically termed as hepatocellular carcinoma [270]. Different animal models have been evaluated over the years to study the symptoms and cures for alcoholic liver diseases. However, the use of animal models for clinical studies experiencing hardship due to ethical issues, species differences, differences present between individual animals, natural aversion of rodents towards alcohol and complicated result interpretation [7, 271, 272]. Thus, a well representative *in vitro* model with functional liver cells becoming more appropriate to study alcoholic liver diseases.

Several two dimensional (2D) *in vitro* models have been discussed previously to analyse the molecular mechanisms involved in alcoholic liver diseases [187, 273]. However, the loss of liver-specific functions of primary hepatocytes at the early stages of culture limits the application of 2D models for *in vitro* studies [274]. To overcome challenges possessed by 2D models, different 3D liver disease models were developed. Lee et al. have been reported a perfusion-based co-culture model of rat primary hepatocytes and rat hepatic stellate cells to study the effect of alcohol treatment on cell viability and functions. This model has shown the importance of co-culture in the recovery of hepatocytes after alcohol injury. The resistance towards hepatocyte apoptosis due to alcohol injury was also improved in co-cultures when compared to monocultures [115]. However, spheroid-based culture models have handling

difficulties and size control issues for long-term culture studies. Gaitantzi et al. reported a combined effect of transforming growth factor- β (TGF- β) and alcohol treatment on the apoptosis of murine primary hepatocytes. On the contrary to Lee et al., this study demonstrated that ethanol alone has no apoptotic effect on hepatocytes in absence of TGF- β , which is a pro-fibrogenic cytokine and upregulates due to alcohol treatment [275]. Though, *in vitro* models based on rodent cells give important insights of alcohol injury, the use of cells from rodent origin decreases its comparability with human cellular behavior due to species differences. To overcome the limitations possessed by the animal cells, Deng et. al. proposed a model with four different types of immortalized human liver cell lines which are HepG2, LX-2, EAhy926 and U937 cell. A range of alcohol treatment from $\sim 3 \mu\text{l/ml}$ to $\sim 9 \mu\text{l/ml}$ was subjected over 48 hours of cell culture study. The use of multiple cell lines from human origin may upgrade the intercellular communications in this proposed model, but it also increases the complexity of the result interpretation due to the incorporation of multiple immortalized cell lines [265]. In the present work, a 3D electrospun random fibrous scaffold was developed by using wet electrospinning of poly(lactic-co-glycolic) acid (PLGA) for long term maintenance of primary human hepatocytes [2]. The co-culture of primary human hepatocytes with hepatic stellate cells further improves the functionality of the human hepatocytes on the fibrous scaffolds modified with essential extracellular matrix proteins. Here we proposed an alcoholic liver disease model with primary human hepatocytes and LX-2 cells which is a human cell line generated by culturing human hepatic stellate cells in low serum media conditions after immortalization with SV40 large T antigen. This cell-scaffold system was treated with different concentrations of alcohol over 4 days to evaluate the effect of cell microenvironment in the maintenance of liver specific functions of human primary hepatocytes upon alcohol injury. A 14 days culture model was designed to study the immediate effect of alcohol injury on human liver cells and the recovery of the injured cells after the withdrawal of alcohol treatment. The alcohol injury was evaluated by studying alcohol metabolizing cytochrome P450 gene expressions i.e. CYP2E1, CYP3A4 and CYP1A2 along with the liver specific functional genes and translational properties of human primary hepatocytes before and after the treatment. The proposed alcoholic liver disease model could be further

evaluated for analysing dose-dependent chemical-induced liver injury and to scan potential drugs for liver fibrosis.

7.2 Materials and methods

7.2.1 Fabrication of alcohol-induced liver fibrosis model

Electrospun 3D scaffolds were surface modified with collagen-I and fibronectin by the chemisorption process (**Figure 7.1**). Four different 3D electrospun scaffolds were used in this study as listed in figure 7.1B . A ratio of 10:1, of primary human hepatocytes and LX-2 cells, were cultured on each scaffold for co-culture studies. For mono-culture studies, 0.1×10^6 cells were seeded on each scaffold. The sandwich culture was used as a gold standard for culturing human liver cells *in vitro*. The cell-scaffold model was treated with 10, 20 and 40 $\mu\text{l/ml}$ of ethanol for 4 consecutive days after 3 days of initial incubation with fresh culture media. After alcohol treatment the recovery of injured liver cells were studied over another 7 days.

Figure 7.1: Schematic presentation of alcoholic liver disease (ALD) model on 3D electrospun scaffolds. (A) Wet electrospinning technique was used to fabricate ALD model with human liver cells. (B) Five different culture models were used with a 10:1 ratio of PHHs to LX-2 to study alcoholic fibrosis *in vitro*.

7.2.2 Flow cytometry

The ratio of PHHs and HSCs at day 3, 7 and 14 were measured by determining the α -SMA expression of LX-2 cells in co-culture groups (**Figure 7.2**). The cells were trypsinized and fixed with 4% paraformaldehyde (PFA) for 15 minutes followed by permeabilization for 30 minutes with 90% methanol. After permeabilization, cells were incubated overnight in incubation buffer with primary antibody (ab7817) at 4°C and for

1 hour in secondary antibody (ab150113) at room temperature. On completion of staining, cells were washed with PBS before proceeding to flow cytometry. BD LSRII flow cytometer (BD Biosciences, Singapore) was used to carry out the experiment. The data obtained from flow cytometry was processed and analysed by using Flowjo software, version 7.6.1.

Figure 7.2: Flow cytometry data on day 3, indicating the percent of HSCs present in the total cell population.

7.2.3 Human Alanine Aminotransferase (ALT) Assay

Human ALT in culture media was quantified by using human ALT ELISA kit (Abcam, Singapore) on day 7 after completion of alcohol treatment. ALT amount was quantified by measuring the absorbance at 450nm using microplate reader.

7.3 Results

7.3.1 Co-culture of primary human hepatocytes and LX-2 cells on 3D electrospun scaffolds

Before alcohol treatment, primary human hepatocytes and LX-2 cells were maintained for 3 days on the gold standard sandwich culture and electrospun 3D PLGA scaffolds. Cell seeding efficiency was measured at day 3 before starting the alcohol treatment to determine the efficiency of the engineered scaffolds (**Figure 7.3**) in promoting attachment of the human liver cells. It was observed that C:F_3:1 culture groups were able to retain significantly higher number of cells compared to all other scaffold types. Seeding efficiency for gold standard sandwich culture was significantly higher compared to the unmodified (C:F_0:0), Collagen-I modified (C:F_1:0) and fibronectin modified (C:F_0:1) scaffolds. However, it was significantly lower compared to the C:F_3:1 groups indicating the effect of favorable microenvironment on increasing cell seeding efficiency in *ex-vivo* cultures, a phenomenon which was discussed in chapter 6 for monoculture of human hepatocytes.

Figure 7.3: Seeding efficiency of PHHs and HSCs on different scaffolds on day 3. $p^{***}<0.001$ compared to other scaffolds on day 3.

On day 3, FESEM technique was used to observe the cell morphology and the interaction between two important liver cells on 3D electrospun scaffolds (**Figure 7.4**). The cell-scaffold interactions were found to be higher on protein modified scaffolds compared to the unmodified 3D PLGA scaffolds. Primary hepatocytes spread along and around the fibers on collagen-I modified fibrous scaffolds (C:F_1:0), whereas cells acquire a more rounded shape on both unmodified (C:F_0:0) and fibronectin modified (C:F_0:1) scaffolds. In C:F_3:1 scaffolds, along with the better attachment of the cells on the electrospun fibers, it also provides better interactions between PHHs and LX-2 cells on the scaffold. Thus, co-presence of two important ECM proteins i.e. Collagen-I and fibronectin on fibrous scaffolds may create superior liver specific microenvironment which results in better cell attachment and intercellular interactions in C:F_3:1 scaffolds.

Figure 7.4: FESEM images of PHHs and LX-2s on unmodified and protein modified scaffolds at day 3, before alcohol treatment. Insets: Showing intercellular interactions.

Functional properties of primary human hepatocytes were analysed by quantifying liver specific gene expressions by using qRT-PCR (**Figure 7.5**). The gene expressions were normalized with a housekeeping gene (E-Cadherin) before normalizing with day 0 (before seeding) gene expression of primary human hepatocyte. Albumin production is the primary characteristic of hepatocytes while cytochrome P450 genes in hepatocytes are responsible for metabolization of different host components. Albumin (ALB) and CYP gene expressions (CYP2C9, CYP3A4, CYP1A2, CYP2E1) were measured at day 3 before alcohol treatment. On C:F_3:1 scaffolds, albumin gene expression was ~7 fold higher than sandwich culture. Similar trend was observed for CYP gene expressions as well. Hepatocytes cultured on C:F_3:1 scaffolds showed ~6 fold higher expression of CYP3A4, ~2 fold higher expression of CYP2C9, ~5 fold higher expression of CYP2E1 and ~6 fold higher expression of CYP1A2 compared to the gold standard sandwich cultures. The albumin and CYP3A4 gene expressions of day 0 hepatocytes (before seeding) and for the hepatocytes cultured on C:F_3:1 scaffolds were found to be comparable, whereas CYP1A2 gene expression was higher in C:F_3:1 culture groups compared to the day 0 hepatocytes. These observations reinforce the role of cell microenvironment in maintaining liver specific functional gene expressions *ex-vivo*.

Figure 7.5: qPCR of liver specific gene expressions: ALB, CYP2C9, CYP3A4, CYP2E1 and CYP1A2 of PHHs cultured on sandwich culture and 3D PLGA fibrous scaffolds on day 3, before alcohol treatment. Gene expressions were normalized by using E-cadherin (E-Cad) as house housekeeping gene before normalizing with day 0 cryopreserved primary human hepatocytes. $p^{***}<0.001$ compared to the sandwich culture.

Albumin secretion of primary human hepatocytes was measured on day 3 to analyse the synthetic properties of the hepatocytes on 3D electrospun scaffolds (**Figure 7.6**). Gold standard sandwich culture showed significantly higher secretion of albumin compared to the cells cultured on C:F_0:0 and C:F_0:1 scaffolds. However, both C:F_1:0 and C:F_3:1 culture groups showed ~3 and ~4 fold higher secretion of albumin on day 3 compared to the sandwich culture. Thus, hepatocytes cultured on C:F_3:1 and C:F_1:0 scaffolds exhibited better synthetic properties due to favorable culture conditions compared to all other culture types. This phenomenon also supported the importance of collagen-I in cell microenvironment in maintaining synthetic properties of primary human hepatocytes *in vitro*.

Figure 7.6: Albumin ELISA was performed to quantify the albumin secretion on day 3 before alcohol treatment. ($p < 0.05$, over sandwich culture group, $n=3$)

Immunostaining was used to visualize the activated phenotype of LX-2 cells (**Figure 7.7**) after 3 days of culture. Alpha-smooth muscle actin (α -SMA) is a marker of the activated phenotype of hepatic stellate cells. Thus, higher expression of α -SMA indicates higher degree of activation in hepatic stellate cells. On TCP all the cells are fully activated as evident from the α -SMA expression (green). On unmodified PLGA scaffolds (C:F_0:0) and collagen-I modified PLGA scaffolds, few α -SMA positive cells were present on day 3. However, the cells in sandwich culture, C:F_0:1 and C:F_3:1 scaffolds were α -SMA negative. This observation may indicate the ability of C:F_0:1 and C:F_3:1 scaffolds in maintaining the quiescent phenotype of hepatic stellate cells. Thus, co-presence of fibronectin and collagen in cell microenvironment may have a significant influence in maintaining cell-phenotype and translational properties of liver cells *in vitro*.

Figure 7.7: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 3 before alcohol treatment. Scale bar = 100 μ m

7.3.2 Alcohol treatment on PHHs-HSCs co-culture

After 3 days of initial co-culture of primary human hepatocytes and LX-2 cells in hepatocyte culture medium, cells were treated with 10, 20 and 40 μ l/ml of alcohol for 4 consecutive days to induce alcohol injury in the proposed model. Different concentrations of alcohol were used to study the intensity of alcohol injury and the corresponding functional response by hepatocytes and stellate cells. As shown earlier, the LX-2 cells, co-cultured with PHHs on tissue culture plate (TCP), acquired activated phenotype even in absence of any fibrosis inducing chemical treatment (**Figure 7.8**). On tissue culture plate, primary human hepatocytes and hepatic stellate cells were cultured and treated with different alcohol concentrations for 4 consecutive days. Hepatic stellate cells on TCP acquired an activated phenotype due to the hardness of the cell microenvironment [9]. Similar phenomenon was observed after 4 days of alcohol treatment of 10:1 PHHs to LX-2 cells co-culture maintained on TCP. α -SMA expression of LX-2 cells were visualized for TCP cultures. It was observed that on TCP, LX-2 cells are α -SMA positive, thus showed an activated phenotype not only for the

alcohol treated co-cultures, but also for the alcohol untreated culture groups (**Figure 7.8B**). Thus, on TCP, LX-2 cells maintained their activated phenotype at all time points irrespective of the presence or absence of alcohol in the culture media. Thus, activated phenotype of LX-2 cells on TCP is more driven by surface hardness rather than alcohol toxicity.

After seeding			Alcohol treatment				Supplementing fresh media without alcohol						
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14

	0 μ l	10 μ l	20 μ l	40 μ l
SC
C:F_0:0
C:F_1:0
C:F_0:1
C:F_3:1

(A)

(B)

Figure 7.8: (A) Cultures were subjected to different concentrations of alcohol over 4 days. (B) Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 of PHHs and LX-2 co-culture on tissue culture plate (TCP); LX-2 cells showed completely activated morphology as evident from α -SMA staining. Scale bar = 100 μ m

Cell viability was evaluated by CCK8 assay after alcohol treatment for all culture groups on 3D scaffolds and sandwich culture (**Figure 7.9**). In sandwich culture, 10 $\mu\text{l/ml}$ alcohol treatment reduces the cell viability, but no further decrease in cell viability was experienced as the alcohol concentration increases from 10 to 40 $\mu\text{l/ml}$. In C:F_0:0 culture group, alcohol treatment did not show apparent effect until the concentration was increased to 40 $\mu\text{l/ml}$. However, generally, C:F_0:0 showed the lowest cell viability compared to all other scaffolds. Thus, ECM protein modification on fibrous scaffold is crucial to support initial cell seeding efficiency and cell viability in subsequent time points. In both C:F_1:0 and C:F_0:1 culture groups, a drop in cell viability was experienced on alcohol treatment to the liver cells. However, cell viability on both C:F_1:0 and C:F_0:1 scaffolds was comparable with the sandwich culture without any alcohol treatment at day 7, which is supporting the hypothesis that ECM protein modification of 3D fibrous PLGA scaffolds can improve viability of the human liver cells *in vitro*. Just like other culture groups, C:F_3:1 culture groups also showed decrease in cell number on alcohol treatment. Despite that, cell viability on C:F_3:1 scaffold was highest amongst all other culture groups with or without alcohol treatment. Thus, C:F_3:1 culture groups were able to maintain better cell viability even under alcohol injury of PHHs in the PHHs and LX-2 co-culture model.

Figure 7.9: Cell viability assay of PHHs and HSCs on different scaffolds on day 7 after completion of alcohol treatment. $p^{***}<0.001$ compared to sandwich culture groups at day 7.

Collagen-I/ Matrigel sandwich culture have been reported widely as a gold standard culture system for human primary hepatocytes. This *in vitro* culture model has been reported to preserve liver specific functional gene expressions such as albumin and CYP2C9 of human primary hepatocytes [276]. Thus, before analysing the gene expressions for 3D cell culture models, we obtained an overview of the effect of alcohol treatment on liver specific functional genes i.e. Albumin, CYP2C9 and the cytochrome P450 genes responsible for alcohol metabolism i.e. CYP2E1, CYP3A4 and CYP1A2 for the PHHs-LX2 co-culture in the sandwich culture model. To analyse the effect of alcohol on liver-specific functional characteristics of the cells cultured in gold standard collagen-I/Matrigel sandwich culture, qRT-PCR was performed on day 7 after completing the alcohol treatment (**Figure 7.10**). The after effect of alcohol treatment or the recovery from alcohol injury was further evaluated on day 14 at the transcriptional level of albumin (ALB) and cytochrome P450 genes i.e. CYP2C9, CYP3A4, CYP2E1 and CYP1A2 which are directly responsible for alcohol metabolism and oxidation of

xenobiotic or endogenous components. 0 μ l/ml alcohol treated cultures on day 7 were used as control for analysing the fold change in respective gene expressions. Hepatocytes cultured in collagen-I/Matrigel sandwich culture showed very little influence of alcohol treatment on albumin gene expressions on day 7 (4th day of alcohol treatment) and no further recovery in albumin gene expression was visible from day 7 to day 14. Thus, it may indicate that the cells are less sensitive towards alcohol treatment in this culture condition. Again, there is no significant increase in albumin gene expression observed for the alcohol untreated cultures (0 μ l/ml) maintained in fresh culture media from day 7 to day 14. Though, a ~3-fold increase in CYP2C9 gene expression was experienced in hepatocytes for 20 and 40 μ l/ml alcohol treatment on day 7 in sandwich culture, the alcohol metabolizing CYP genes i.e. CYP3A4, CYP2E1 and CYP1A2 show very little increase in expression only after 40 μ l/ml of alcohol treatment. The increase in CYP gene expression upon alcohol treatment was insignificant for CYP3A4 and CYP1A2, but it was significant for CYP2E1 ($p < 0.001$). For, 10 and 20 μ l/ml alcohol treatment, all CYP3A4, CYP2E1 and CYP1A2 genes were downregulated which may be due to less efficiency of sandwich cultures in maintenance of CYP activity upon alcohol injury at day 7. On day 14, alcohol treated cultures showed reduced level of CYP gene expressions, which was an expected observation as the alcohol treatment was withdrawn from the system. This observation indicates the less efficiency of sandwich culture group in preserving liver-specific functional properties of human primary hepatocytes under alcohol treatment.

Figure 7.10: Albumin (ALB) and cytochrome P450 gene expressions of PHHs in sandwich culture: Ethanol exposure does not affect ALB expressions. Alcohol metabolism related genes i.e. CYP3A4, CYP2E1 and CYP1A2 showed no significant increase over alcohol treatment. 0 µl alcohol treatment samples considered as control. $p^{***}<0.001$ compared to 0 µl alcohol treatment and $p^{###}<0.001$ compared to day 7.

To further visualize the alcohol injury in sandwich cultures, immunostaining of α -SMA was performed on day 7 (**Figure 7.11**). Unlike CYP gene expressions observed from qRT-PCR assay, α -SMA positive cells were found to be increased on increasing alcohol concentration. Thus, in sandwich culture, stellate cells were able to retain the characteristics of alcohol injury which was less evidenced in primary human hepatocytes.

Figure 7.11: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 of PHHs and LX-2 in sandwich culture; Alcohol treatment increases α -SMA expression as LX-2 acquires more activated phenotype.

The analysis of liver-specific functional properties of PHHs on unmodified 3D PLGA scaffolds (C:F_0:0) were performed on day 7, just after alcohol treatment and on day 14 to visualize the efficiency of the C:F_0:0 in maintaining functional hepatocytes *in vitro* under alcohol injury (**Figure 7.12**). The albumin gene expressions of PHHs cultured on C:F_0:0 scaffolds decreased from day 7 to day 14 even in absence of any alcohol treatment. This observation may be supporting the fact that PLGA scaffolds, in absence of any biologically active molecules are less effective in maintaining liver specific functionality of primary human hepatocytes *in vitro*. A gradual decrease in albumin gene expression was visible in C:F_0:0 culture groups on increasing concentration of alcohol. Thus, alcohol treatment suppresses the activity of the albumin gene which was not recovered over time as the cultures were maintained from day 7 to day 14. Cytochrome P450 genes which are directly responsible for alcohol metabolism i.e. CYP3A4, CYP2E1 and CYP1A2 showed a gradual increase in gene expressions on increasing alcohol concentration in the media. However, CYP2E1 and CYP1A2 genes become less responsive towards alcohol toxicity as the alcohol concentration in culture media increases to 40 $\mu\text{l/ml}$ which may be very high to maintain CYP2E1 and CYP1A2 gene expressions on C:F_0:0 scaffolds on day 7. CYP2C9 gene expression also shown significant increase after 10 $\mu\text{l/ml}$ alcohol treatment on day 7. However, the trend was not very significant, once the alcohol concentration increases to 20 and 40 $\mu\text{l/ml}$. Thus, on C:F_0:0 scaffolds, hepatocytes loses its functional gene expression as the alcohol concentration increases over 10 $\mu\text{l/ml}$ to 40 $\mu\text{l/ml}$. Albumin, CYP2C9 and CYP3A4 which are liver-specific functional genes, showed no improvement in expression as the culture was maintained till day 14. However, a significant increase in CYP2E1 and CYP1A2 gene expression was experienced for alcohol untreated cultures over day 7 to day 14 on C:F_0:0 scaffolds. Thus, a combined effect of 3D fibrous environment with the absence of bioactive microenvironment was found in C:F_0:0 scaffolds for maintaining PHHs/LX-2 co-culture under healthy and disease conditions.

Figure 7.12: Albumin (ALB) and cytochrome P450 gene expressions of PHHs in C:F₀:0: Unlike sandwich culture, ethanol exposure affects ALB expressions in C:F₀:0 cultures. Cytochrome P450 genes i.e. CYP3A4, CYP2E1 and CYP1A2 was affected by the alcohol treatment an upward trend for 10 and 20 μl/ml alcohol on unmodified PLGA fibrous scaffolds. p***<0.001 compared to 0 μl alcohol treatment and p###<0.001 compared to day 7.

Immunostaining of α -SMA on C:F₀:0 culture groups supports the observation obtained from gene expression analysis (**Figure 7.13**). On adding alcohol, α -SMA expression increased and showed a gradually upregulating trend from 0 to 40 μl/ml of alcohol treatment. Thus, alcohol treatment increased α -SMA positive cells in the C:F₀:0 scaffolds and induced fibrosis in the PHHs/LX-2 co-culture model. The minimal α -SMA expression of LX-2 cells on C:F₀:0 scaffolds in absence of alcohol (0 μl/ml of alcohol treatment) also proved that these 3D electrospun scaffolds with liver ECM mimicking architecture was able to maintain the quiescent phenotype of stellate cells when compared to the TCP cultures (**Figure 7.8B**) where LX-2 cells shown activated phenotype at all culture conditions.

Figure 7.13: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 of PHHs and LX-2 cells cultured on C:F_0:0 scaffolds; Alcohol treatment increases α -SMA expression as LX-2 acquires more activated phenotype.

C:F_1:0 scaffolds were found to be effective to maintain functional primary human hepatocytes on 3D electrospun scaffolds in absence of alcohol injury as we have discussed in chapter 4 and chapter 5. To visualize its efficiency in preserving liver-specific functional genes upon alcohol injury, qRT-PCR was performed on day 7 and day 14 for PHHs-LX-2 co-cultures (**Figure 7.14**). In C:F_1:0, i.e. Collagen-I modified PLGA scaffolds, albumin gene expression increases ~2.5-folds from day 7 to day 14 for 0 μ l/ml of alcohol treated group. Thus, collagen-I modified fibrous PLGA scaffolds providing 3D microenvironment showed better maintenance of albumin gene expression over time. For 10 μ l/ml alcohol treatment, the albumin gene expression was maintained and remain similar to the 0 μ l/ml alcohol treatment on day 7. However, on increasing alcohol concentration from 10 to 20 and 40 μ l/ml, the albumin gene expression decreased, indicating that alcohol treatment affects synthetic properties of primary human hepatocytes. Among cytochrome P450 genes, CYP2C9, CYP2E1 and CYP1A2 expressions were sharply increased from 0 to 10 μ l/ml alcohol treatment. However, these gene expressions shown a drastic decrease as the alcohol concentration increases to 20 and 40 μ l/ml. CYP3A4, gene expression however was maintained for all alcohol concentrations on the C:F_1:0 scaffolds which provides a 3D, liver ECM mimicking microenvironment to the primary human hepatocytes. On day 7 to 14; albumin, CYP2C9, CYP2E1 and CYP1A2 gene expressions were maintained and the expression of CYP3A4 increase significantly in the alcohol untreated culture groups, whereas in alcohol treated groups a decrease in albumin and CYP gene expression was observed from day 7 to day 14 due to the alcohol toxicity in PHHs on C:F_1:0 scaffolds.

Figure 7.14: Albumin (ALB) and cytochrome P450 gene expressions of PHHs in C:F₁:0: 20 and 40 µl/ml ethanol exposure affects ALB expressions. Cytochrome P450 gene expressions (CYP2C9, CYP3A4, CYP2E1, CYP1A2) increased for 10 µl/ml alcohol treatment. However, PHHs seemed to loss liver specific functional gene expressions for higher concentration of alcohol treatment on C:F₁:0 scaffolds. $p^{***}<0.001$ compared to 0 µl alcohol treatment and $p^{###}<0.001$ compared to day 7

In immunostaining of α -SMA on C:F₁:0 culture group (**Figure 7.15**), it was visualized that in absence of alcohol in the culture media, LX-2 cells showed a quiescent phenotype thus remain α -SMA negative. On treatment with alcohol, a gradual increase in α -SMA positive cells were observed on C:F₁:0 scaffolds due to increase in activated phenotype of LX-2 cells.

Figure 7.15: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 on C:F_1:0 scaffolds; Alcohol treatment increases α -SMA expression as LX-2 acquires more activated phenotype with increasing alcohol concentration.

Liver specific gene expressions were further evaluated for the PHHs cultured on C:F_0:1 scaffolds on day 7 and day 14. In C:F_0:1 culture groups, albumin gene expression was maintained as the alcohol concentration was increasing from 0 to 10 to 20 $\mu\text{l/ml}$ (**Figure 7.16**) on day 7. However, on further increase in alcohol concentration to 40 $\mu\text{l/ml}$, a decrease in albumin gene expression was experienced which is indicating that 40 $\mu\text{l/ml}$ alcohol concentration is too high for the PHHs to maintain their albumin gene expression on C:F_0:1 scaffolds. Similar trend was observed on day 7 for CYP2C9, CYP2E1 and CYP1A2 genes expressions. An increase in transcriptional level was observed for CYP2C9, CYP2E1 and CYP1A2 genes when the alcohol concentration was increased from 0 to 20 $\mu\text{l/ml}$. However, for 40 $\mu\text{l/ml}$ alcohol treatment, a decrease in the transcriptional level for CYP2C9, CYP2E1 and CYP1A2 was observed which may be attributed to the loss of liver specific functions at higher concentration of alcohol in *ex-vivo* culture on C:F_0:1 scaffolds. CYP3A4 gene expression of PHHs was maintained for all the concentrations of alcohol treatment on C:F_0:1 scaffolds. However, the effect of alcohol treatment was not very much evident from the CYP3A4 gene expressions, thus C:F_0:1 scaffolds may show less efficiency to study the effect of alcohol injury on cytochrome P450 genes under the given culture conditions. However, an improved albumin and CYP gene expression from day 7 to day 14 in alcohol untreated cultures indicates the efficiency of the C:F_0:1 scaffolds for maintain functional PHHs in the *in vitro* culture.

Figure 7.16: Albumin (ALB) and cytochrome P450 gene expressions of PHHs in C:F_0:1: Albumin gene expression increases a little on increasing alcohol concentration. However, except CYP3A4, other CYP gene expressions i.e. CYP2C9, CYP2E1 and CYP1A2 were steady and increased till 20 µl/ml alcohol treatment. $p^{***}<0.001$ compared to 0 µl alcohol treatment and $p^{###}<0.001$ compared to day 7.

To further evaluate the effect of alcohol on LX-2 cells in C:F_0:1 cultures, immunostaining of α -SMA was performed on day 7 after alcohol treatment (**Figure 7.17**). A gradual increase in α -SMA expression was observed for the LX-2 cells cultured on C:F_0:1 scaffolds as the alcohol concentration increase to 40 µl/ml from 0 µl/ml in the culture media. However, no α -SMA positive cells were observed for 0 µl/ml i.e. alcohol untreated groups on day 7 indicating the efficiency of C:F_0:1 scaffolds to maintain quiescent phenotype of LX-2 cells in absence of any fibrosis inducing agents.

Figure 7.17: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 of PHHs and LX-2 cells cultured on C:F_0:1 scaffolds; Alcohol treatment increases α -SMA expression as LX-2 acquires more activated phenotype.

Finally, the effect of alcohol treatment was analyzed for C:F_3:1 culture groups which was found to be efficient in maintaining long-term culture of human hepatocytes *in vitro* (chapter 6). The Albumin and cytochrome P450 gene expressions were evaluated for the PHHs cultured on C:F_3:1 scaffolds (**Figure 7.18**). On these scaffolds (C:F_3:1), albumin gene expression was maintained till 10 μ l/ml alcohol treatment. However, for 20 and 40 μ l/ml alcohol treatment, albumin gene expression downregulated as higher concentration of alcohol affects the transcriptional level of the albumin gene. On day 14, in alcohol untreated culture group, albumin gene expression on C:F_3:1 scaffolds increased by ~2.5-fold, CYP2C9 gene expression increased by ~1.5-fold, CYP3A4 gene expressions increased by ~2.5-fold and CYP1A2 gene expression increased by ~5-fold over day 7. CYP2E1 gene expression also showed an upward trend from day 7 to day 14 on C:F_3:1 scaffolds in absence of alcohol in the culture medium. Thus, C:F_3:1 scaffolds are found to be superior in maintaining liver specific functional gene expressions for a long term culture of human hepatocytes which was also discussed in chapter 6. On alcohol treatment, as the alcohol concentration increased from 0 to 40 μ l/ml, all the CYP gene expressions i.e. CYP2C9, CYP3A4, CYP2E1 and CYP1A2 showed a gradual increase at the transcriptional level on C:F_3:1 scaffolds. Again, for alcohol untreated culture groups an increase in albumin and CYP gene expressions was observed from day 7 to day 14 on C:F_3:1 scaffolds. Thus, C:F_3:1 scaffolds were able to retain the liver specific gene expressions of PHHs under all the experimental conditions indicating that the features of alcohol injury was visible on cells cultured on C:F_3:1 as would be in *in vivo* conditions.

Figure 7.18: Albumin (ALB) and cytochrome P450 gene expressions of PHHs in C:F₃:1: Albumin gene expression showed a gradual decrease on increasing alcohol concentration. All CYP expressions showed an upward trend on increasing concentration of alcohol. $p^{***}<0.001$ compared to 0 μ l alcohol treatment and $p^{###}<0.001$ compared to day 7.

Immunostaining of LX-2 cells on C:F₃:1 scaffolds shows an increase in α -SMA expressions upon treating the cells with alcohol (**Figure 7.19**). However, on C:F₃:1 scaffolds, for 20 and 40 μ l/ml alcohol treatment, α -SMA expressions was much less pronounced compared to the other cultures groups containing equal amount of alcohol in the culture media. This observation may indicate that, C:F₃:1 scaffolds are better in maintaining quiescent phenotype of LX-2 cells on increasing alcohol concentration when compared to all other scaffolds under alcohol toxicity.

Figure 7.19: Immunostaining of α -SMA (Green) and Nucleus (DAPI) on Day 7 of PHHs and LX-2 cells cultured on C:F_3:1 scaffolds; Alcohol treatment increases α -SMA expression as LX-2 acquires more activated phenotype. Little enhancement of α -SMA expression was experienced as the alcohol concentration increases from 10 to 40 μ l/ml.

Hepatocytes produce Alanine aminotransferase (ALT), which is present in very low concentration in the body serum for the healthy liver. However, ALT concentration in body serum increases on liver injury or during liver diseases [277]. To validate the proposed model, we quantified the amount of ALT present in the culture media on day 7 after completion of alcohol treatment (**Figure 7.20**). In collagen-I/Matrigel sandwich culture, ALT level was increased on alcohol treatment, though no significant change is ALT production was observed as the alcohol concentration was increased from 0 to 40 $\mu\text{l/ml}$. In C:F_0:0, C:F_1:0 and C:F_0:1 culture models, ALT level in the culture media was very low and remain undetectable, whereas in C:F_3:1 culture groups, a gradual increase in ALT level in the culture media was observed on increasing the alcohol concentration from 0 to 40 $\mu\text{l/ml}$. Again, this indicates that the C:F_3:1 scaffolds were able to maintain cells such that they are responsive to alcohol trigger as would the native cells in *in vivo* conditions. This make the scaffolds good candidates for liver disease models.

Figure 7.20: Alanine aminotransferase (ALT) release by alcohol injured primary hepatocytes were measured for all culture groups on day 7. $p^{***}<0.001$, compared to the 0 $\mu\text{l/ml}$ alcohol treated culture group.

The synthetic properties of primary hepatocytes were analyzed by quantifying albumin secretion on day 7 after 4 days of alcohol treatment for PHHs/LX-2 co-cultures (**Figure 7.21**). It was observed that alcohol treatment affects the albumin secretion efficiency of the primary hepatocytes for all culture types. In absence of alcohol treatment i.e. for 0 μ l/ml alcohol treatment, both C:F_1:0 and C:F_3:1 cultures shown ~1.3 fold and ~1.6 fold higher secretion of albumin compared to the gold standard collagen-I/Matrigel sandwich culture. A gradual decrease in albumin secretion was experienced with increasing alcohol concentration in all the cultures. However, C:F_3:1 scaffolds were able to maintain higher albumin secretion by the hepatocytes compared to the sandwich cultures after the alcohol treatment. For both 20 and 40 μ l/ml alcohol treatment a ~2 fold higher secretion of albumin was observed for C:F_3:1 scaffolds compared to the sandwich culture.

Figure 7.21: Albumin ELISA on Day 7 after alcohol treatment; Alcohol treatment reduces albumin secretion by PHHs. However, cells cultured on C:F_3:1 scaffolds showed significantly higher secretion at all culture conditions. * $p < 0.05$ and *** $p < 0.001$ compared to sandwich culture.

Thus, C:F_3:1 scaffold was able to maintain liver-specific functional properties of primary human hepatocytes in the *in vitro* culture model either in the presence or absence of alcohol injury. This scaffold-cell assembly thus can also be used to study the effects of other fibrosis causing chemicals or cytokines on the human liver cells *in vitro*.

7.4 Discussion

In vitro liver disease models are gradually becoming an important tool to study pathophysiological characteristics of liver diseases. A cell culture platform that can

support translational and transcriptional properties of liver cells needs to mimic the physical and chemical properties of the *in vivo* cell microenvironment. Several such approaches have been reported previously by many researchers using different cell-scaffold assemblies [278, 279]. In the current study, a 3D electrospun PLGA scaffold modified with collagen-I and fibronectin to support liver-specific functional properties of PHHs and hepatic stellate cells was developed to fabricate *in vitro* alcoholic liver disease model. A pre-optimized ratio of primary human hepatocytes and stellate cells (10:1) (chapter 5) was seeded on each scaffolds to retain functional properties of primary human hepatocytes in a PHHs/HSCs co-culture. Different concentrations of alcohol were added into the culture medium to investigate the effect of a) alcohol concentration, b) multiple ECM protein modification on electrospun scaffolds and finally c) the effect of 3D fibrous microenvironment on liver-specific functional properties of primary human hepatocytes. Seeding efficiency quantification was performed on day 3 to evaluate the degree of cell attachment on each scaffold. Though unmodified and single protein modified scaffolds showed lower seeding efficiency due to removal of non-adherent cells, C:F₃:1 scaffold which has both collagen-I and fibronectin simultaneously, were able to retain a significantly higher number of seeded cells compared to all other cultures. Thus, chemisorption of electrospun fibers with collagen-I and fibronectin provides a better microenvironment to the liver cells, which was responsible for improved cell seeding efficiency on day 1 (**Figure 7.3**). A similar approach was reported by Grant et al. in a recent publication where biofunctionalization was performed on electrospun poly-L-lactic acid (PLA) fibrous mat to coat the scaffold surface with cell-produced ECM proteins. This biofunctionalized scaffold was used to culture HepG2 cells. However, this technology gives a 2D cell microenvironment where the composition of ECM proteins present on the spun scaffolds was undefined [280]. The same research group also reported the fabrication of a bioengineered scaffold, prepared by conventional electrospinning of the decellularized human liver ECM and PLA blend for improving the *in vitro* microenvironment of human hepatocytes. The challenges exist with using decellularized human liver are the variation in ECM composition and faster enzymatic degradation [281]. Moreover, the small pore size of the electrospun fibrous mat restricts the cellular infiltration thus provides a more 2D

microenvironment which is very much different from the *in vivo* 3D microenvironment of the liver cells. In this study, we used 3D fibrous scaffolds modified with a known amount of essential ECM proteins to preserve liver-specific functional characteristics of both PHHs and LX-2 cells. C:F_3:1 scaffold also shown better intra and intercellular interactions between PHHs and LX-2 cells compared to unmodified or single protein modified scaffolds which show the importance of the co-presence of both the proteins to maintain functional liver cells *in vitro* (**Figure 7.4**). To analyse the effect of alcohol treatment we used three different concentrations of alcohol which are 10 μ l/ml, 20 μ l/ml and 40 μ l/ml. Alcohol toxicity in the liver results in activation of the hepatic stellate cells which leads to scar tissue formation by producing excessive extracellular matrix, mainly fibrous collagen-I. These events on alcohol injury will lead to apoptosis of the hepatocytes. In sandwich culture, alcohol treatment showed a minimal effect on the liver-specific functional characteristics of primary human hepatocytes, though activated phenotype of LX-2 cells was enhanced on increasing alcohol concentration (**Figure 7.10**, **Figure 7.11**). Thus, collagen-I/Matrigel sandwich culture showed less efficiency to maintain pathophysiological functions of primary human hepatocytes upon alcohol treatment. In unmodified 3D scaffolds and single protein modified scaffolds, alcohol treatment reduces the albumin gene expression and increases the CYP expressions for 10 and 20 μ l/ml alcohol treatment (**Figure 7.12**). However, a loss in functional properties was observed for 40 μ l/ml alcohol treatment in unmodified and single protein modified scaffolds. Thus, 40 μ l/ml alcohol concentration was found to have an adverse effect on the PHHs liver-specific functional properties when cultured on unmodified or single protein modified scaffolds. However, a gradual increase in CYP expressions were visible on 10 and 20 μ l/ml of alcohol treatment in C:F_0:0 (**Figure 7.12**), C:F_1:0 (**Figure 7.14**) and C:F_0:1 (**Figure 7.16**) scaffolds which was not observed for the gold standard sandwich culture (**Figure 7.10**) indicating the effect of 3D fibrous microenvironment in maintaining functional properties of PHHs *ex-vivo*. Among all culture groups, only C:F_3:1 scaffold was able to maintain both albumin (ALB) and CYP gene expressions (CYP2C9, CYP3A4, CYP2E1, and CYP1A2) for 40 μ l/ml alcohol treatment (**Figure 7.18**). Thus, liver-specific gene expressions of PHHs were preserved in C:F_3:1 scaffold at all concentrations of alcohol treatment. The

response of LX-2 cells towards alcohol injury was found to be consistent for all scaffold types, i.e. a gradual increase in α -SMA expression was visible from immunostaining images with increasing alcohol concentration. It was reported previously that ethanol interferes with retinoid metabolism and enhances the release of fibrogenic cytokines such as collagen-I, transforming growth factor- β 1 from hepatic stellate cells [282]. Ethanol also perpetuates the activation of HSCs by remodeling of liver ECM. Activated HSCs remain α -SMA positive, as it was observed for the cultures maintained on TCP (**Figure 7.8**) where at all the conditions, the activated phenotype of HSCs was observed due to surface hardness of the culture plate [9]. However, LX-2 cells remain α -SMA negative in both sandwich culture and for the cultures maintained on 3D electrospun scaffolds for alcohol untreated cultures on day 7. Thus, 3D fibrous scaffolds were able to maintain the quiescent phenotype of hepatic stellate cells in absence of alcohol injury. On adding alcohol to the culture media, a gradual increase in α -SMA expression of LX-2 cells shows the role of ethanol in initiating activation of hepatic stellate cells. To further investigate the effect of alcohol injury, ALT release was examined for all culture types after completion of alcohol treatment (**Figure 7.20**). Under *in vivo* conditions, ALT concentration increases with increasing alcohol injury. The same phenomenon was experienced for the PHHs/ LX-2 co-cultures maintained in C:F_3:1 scaffold, however, this phenomenon was absent for other scaffold conditions indicates that C:F_3:1 scaffold create better liver-specific microenvironment, which was less efficient in unmodified or single protein modified scaffolds for human liver cells. Thus, C:F_3:1 scaffold with functional human liver cells can be used as a model to study pathophysiological characteristics of alcoholic liver injury. This model is also anticipated to have further potential to examine the effect of other fibrosis causing materials.

7.5 Conclusion

In this study, we reported the fabrication and characterization of an *in vitro* liver disease model by using essential and minimal ECM protein modified 3D electrospun nanofibrous PLGA scaffolds. A co-culture of PHHs with human hepatic stellate cells was characterized by evaluating functional and pathophysiological activities at

different cell culture conditions under alcohol treatment. This *in vitro* model was found to give better insights into cellular functions with increasing alcohol concentration compared to the gold standard sandwich culture where PHHs lose their liver-specific functions under alcohol treatment. Further scopes in introducing other liver cell types or scaffold functionalization are also present in this reported culture model. Thus, the proposed *in vitro* model has the potential to be used as disease model for drug-screening and to facilitate the study of hepatotoxicity and ALD-related mechanisms.

Chapter 8

Summary and Future Scopes

This chapter includes the summary of the thesis and recommendations for future direction. Section 8.1 summarizes the work with respect to the research objectives and discussed how the three defined aims were achieved. The results obtained during this study gives important insights for development of in vitro liver disease model by using electrospun 3D fibrous scaffolds and human liver cells. Section 8.2 includes future recommendations of this work for improvisation of the proposed disease model and discussed how this model will be beneficial to study different types of liver diseases.

8.1 Summary

The overall aim of this study is to create an *in vitro* three-dimensional liver disease model with human liver cells by closely mimicking the physical and chemical characteristics of the liver extracellular matrix. We have reported the fabrication of electrospun nanofibers scaffolds by wet electrospinning process and chemisorption of the fiber surface with suitable macromolecular proteins to mimic the physical and chemical characteristics of the native liver extracellular matrix [2]. This dissertation is divided into three major aims: 1) Fabrication and characterization of 3D electrospun fibers followed by surface modification with biologically active molecules; 2) Investigation of fiber surface modification and co-existence of collagen-I and fibronectin on viability and functionality of human hepatocytes; 3) Evaluation of the effect of alcohol treatment on the proposed scaffold-cell culture model to study alcoholic liver fibrosis *in vitro*. An interdisciplinary approach was applied for liver tissue engineering by combining a biodegradable and biocompatible polymer with biologically active macromolecular proteins. Cell microenvironment was tuned by modification of fibrous scaffolds with one or more extracellular matrix proteins for fabricating an optimum microenvironment to preserve functional liver cells *in vitro*. This engineered tissue can be applied to fabricate liver disease model which will contribute largely to scan toxins or drugs for human liver cells *in vitro* under normal or diseased conditions.

To fulfill the first aim, which is fabrication and characterization of 3D electrospun fibers followed by surface modification with biologically active molecules, the wet electrospinning technique was applied. This newly evaluated electrospinning technique was used previously by our lab to fabricate scaffolds for skin tissue engineering [255]. Poly(lactic-co-glycolic)acid, a biodegradable and biocompatible polymer, has been used to prepare scaffolds for several tissue engineering applications [4, 283, 284]. We optimized the parameters of the electrospinning technique to prepare 3D electrospun nanofibrous scaffolds with optimum porosity and pore size which was close to the pore size of the native liver extracellular matrix. The pore size of the scaffold plays a vital

role in cell-cell communication and cell infiltration inside the scaffold. A porous scaffold creates a 3D cellular microenvironment whereas, in non-porous or scaffolds with small pore sizes, cells remain on the top of the scaffold, thus created a more 2D microenvironment. We described this phenomenon by comparing electrospun fibrous mat, prepared by traditional electrospinning technique with 3D porous scaffold prepared by wet electrospinning technique. In our work, we found that the wet electrospinning technique improved the pore size of the electrospun PLGA fibers by ~87 – 126 % over the PLGA spun fibers obtain by conventional electrospinning technique. Chemisorption process was applied to modify the fiber surface with collagen-I or fibronectin at different protein concentrations. Collagen-I and fibronectin are important components of liver ECM, previously used to create a biologically active microenvironment for liver cells [140, 252, 285, 286]. However, the differential effect of these two essential proteins on human primary hepatocytes, cultured on the liver ECM mimicking fibrous network was still to be explored. The chemisorption process was standardized to obtain maximum surface modification and retention of the ECM proteins on PLGA fibers. Further increase in protein concentration was not able to induce higher protein retention on the scaffold surface on completion of the chemisorption process. Again, chemisorption of 3D wet electrospun PLGA scaffolds found to have no significant effect on the scaffold porosity (> 90 %), pore size (~10 – 45 μm) and fiber diameter (~1000 nm). This protein modified 3D PLGA scaffolds were used in the later sections of the dissertation to study on the efficiency of the same in maintaining liver-specific functions of the human hepatocytes.

The second aim of this dissertation was “investigation of fiber surface modification and co-existence of collagen-I and fibronectin on viability and functionality of human hepatocytes”. We cultured primary human hepatocytes on both collagen-I and fibronectin modified PLGA scaffolds to achieve this aim. Collagen-I/Matrigel sandwich culture, known as the gold standard for preserving liver-specific functional properties of the human primary hepatocytes *in vitro* was used as control. Our results showed that protein modification on the fibrous scaffolds reinforces the liver-specific microenvironment *in vitro*, therefore liver-specific functions of PHHs were preserved

better on protein modified scaffolds compared to the unmodified 3D fibrous electrospun PLGA scaffolds. Collagen-I modified electrospun nanofibrous 3D PLGA scaffolds was found to be superior to gold standard sandwich culture in preserving *in vitro* functional maintenance of primary human hepatocytes. These observations suggested that the presence of collagen-I on a 3D scaffold is essential for *in vitro* maintenance of functional hepatocytes. There are many articles suggested the positive influence of both collagen-I and fibronectin in creating a suitable microenvironment for hepatocytes *in vitro*. Thus, to further improve the efficiency of the proposed synthetic platform we fabricated electrospun nanofibrous PLGA scaffolds by modifying the scaffold surface with both collagen-I and fibronectin. Collagen-I and fibronectin are macromolecules that have long protein chains. Thus, the efficient distribution of these two essential ECM proteins on the scaffold surface found to be challenging. Addition of these two ECM proteins sequentially results in protein accumulation at different locations on the scaffold surface. However, the addition of the protein mixture results in more homogeneous distribution and less protein accumulation (**Figure 8.1**).

Figure 8.1: FESEM image of C:F_3:1 (A) Both collagen and fibronectin added together to modify the fiber surface; (B) Collagen is added followed by fibronectin to modify the fiber surface; (C) Fibronectin is added followed by collagen to modify the surface of the PLGA fibers.

It was also observed that different ratio of these two individual protein results in the different distribution pattern on the uneven fiber surface during the chemisorption process. We found that at optimum concentrations of collagen-I and fibronectin, a homogeneous distribution of both the proteins on the fibrous scaffolds can be achieved. This phenomenon also improved the efficiency of the chemisorption process which

results in maximum total protein content on the scaffold at the optimum protein ratio which was 3 to 1 of collagen-I to fibronectin. The efficiency of the optimized ECM protein modified scaffolds in maintaining functional human hepatocytes *in vitro* was evaluated by culturing Huh-7.5 cells for 28 days. Along with 3:1 collagen-I to fibronectin modified scaffolds, 1 to 1 and 9 to 1 collagen-I to fibronectin modified scaffolds along with the only collagen-I modified, only fibronectin modified and unmodified 3D electrospun fibrous scaffolds were also tested for functional maintenance Huh-7.5 cells over 28 days of culture. This study suggested that co-presence of collagen-I with fibronectin at the optimum ratio in 3D electrospun nanofibrous scaffolds improved translational and transcriptional properties of human hepatocytes in the *in vitro* culture. Thus, the co-presence of two essential extracellular matrix proteins on this synthetic nanofibrous scaffold surface showed a synergistic influence on the functional properties of the liver cells. Observations also revealed that, the homogeneous presence of two ECM proteins further improved the liver-specific functions of the Huh-7.5 cells. By accomplishing this aim, we established that i) It is possible to achieve a homogeneous distribution of macromolecular ECM proteins on the uneven 3D fibrous scaffolds by using optimum concentration of the macromolecular proteins for chemisorption process; ii) Surface modification of the PLGA fibrous scaffolds with biologically active macromolecular proteins can influence positively the *in vitro* maintenance of functional hepatocytes; and finally, iii) Homogeneously chemisorbed fibers with essential ECM proteins can influence liver-specific characteristics of human hepatocytes under *in vitro* culture conditions.

The observations gathered on completion of aim 2 motivated us to find its application in the fabrication of an *in vitro* disease model with human liver cells to scan toxins or drugs related to human liver diseases. As a proof of concept, we fabricated an alcoholic liver disease model by using this essential ECM protein modified 3D electrospun scaffolds which bring us to the third aim, which was "Evaluation of the effect of alcohol treatment on the proposed scaffold-cell culture model to study alcoholic liver fibrosis *in vitro*". To fulfill this aim, primary human hepatocytes which are the main parenchymal liver cell and human hepatic stellate cells which are the key initiator of

liver diseases by causing fibrosis on liver injury was co-cultured on the protein modified electrospun scaffolds. A combination of these two major human liver cells were used to establish an alcoholic liver disease model for reducing the complexity of the system and for a more basic understanding of the effect of alcohol on the proposed *in vitro* culture model.

Before investigating the effect of alcohol on human primary hepatocytes and human hepatic stellate cells, the ratio of primary human hepatocytes and human hepatic stellate cells was optimized by comparing hepato-specific functional properties of PHHs on collagen-I modified 3D electrospun scaffolds at different ratios of PHHs and HSCs. A 10 to 1 PHHs to HSCs was found to be optimum and is able to retain functional properties of human hepatocytes over 14 days of culture. This optimized ratio of hepatocytes and stellate cells was used to further evaluate the effect of alcohol injury in the proposed 3D model. A series of alcohol concentration was tested for hepatocytes-stellate cells co-culture not only on the optimized ECM modified scaffold which contains 3 to 1 collagen-I to fibronectin but also for only collagen-I modified, only fibronectin modified and ECM unmodified scaffolds. Sandwich cultures, which is considered as the gold standard for *in vitro* culture of primary human hepatocytes were used as a control in this study. A differential effect of alcohol treatment on PHHs and Lx-2 cells was observed for different scaffold conditions. C:F_3:1 scaffold which was optimized for better long-term maintenance of functional hepatocytes were able to show maximum preservation of hepatocytes functions upon alcohol treatment over other scaffold conditions. Thus, cell microenvironment for the *in vitro* cultures is also found to be crucial under disease conditions. Increasing alcohol concentration showed a detrimental effect on the synthetic properties of primary human hepatocytes, such as albumin production. CYP enzymatic activity was preserved and showed the expected upregulation under hepatocytes injury when cells cultured in the favorable microenvironment. Cell-microenvironment was also found to have a profound effect on cell-cell and cell-scaffold interactions which is further responsible for retention of cellular functions *in vitro*. Overall, this study gives us insights of i) the effect of alcohol treatment on liver-specific functional properties of primary hepatocytes; ii) the effect

of increasing alcohol concentration at the transcriptional level in primary human hepatocytes; iii) phenotypic alteration of human hepatic stellate cells upon alcohol treatment; and most importantly iv) the effect of physically and chemically *in vivo* mimicking microenvironment to control *in vitro* functions of primary human hepatocytes at normal or disease conditions.

The findings of this dissertation give valuable information about the dependence of functional properties of human liver cells on cell-microenvironment for the *in vitro* studies. In this work, we reported a tunable microenvironment by using electrospun PLGA nanofibers. This cell-scaffold assembly has further scopes of improvisation by introducing other major liver cells or application of a combination of essential ECM proteins to modify nanofibrous 3D scaffolds, which could be specific for studying specific liver diseases.

8.2 Future recommendations

Based on the outcomes from this work, we recommend the following future directions.

8.2.1 Improvisation of the liver disease model on 3D electrospun nanofibrous scaffolds

In this study, we demonstrated the liver ECM mimicking characteristics of the electrospun nanofibrous scaffolds. Our study also confirms that the 3D fibrous network improves the *in vitro* cell-microenvironment of hepatocytes over the 2D culture on fibrous mat prepared by conventional electrospinning process. Surface modification of the 3D scaffolds with important ECM proteins was found to be crucial for the long-term viability of human liver cells *in vitro*. In the reported study, the optimization of collagen-I and fibronectin modification on wet electrospun 3D scaffolds have been discussed. Other than collagen-I and fibronectin, liver ECM consists of many other ECM proteins such as collagen-IV, laminin, etc. To introduce all the components of liver ECM researchers have used blending or coating of native liver extracellular matrix

proteins on synthetic polymeric materials or use a coat of the native decellularized liver matrix on tissue culture plate [278, 287]. However, this approach is not able to give a controlled amount and composition of essential ECM proteins on the scaffold surface. Thus, the effect of individual ECM proteins in regulating liver-specific functional properties of the human liver cells is difficult to evaluate. There is also present batch to batch variation in the composition of the native decellularized liver ECM. By using the proposed chemisorption process, it is possible to further optimize the amount of other minimal essential ECM proteins for modification on the electrospun scaffolds. It may further improve the cell microenvironment for *in vitro* studies on liver ECM mimicking electrospun nanofibrous scaffolds.

Another approach we discussed in this dissertation is the use of the co-culture of primary human hepatocytes and human hepatic stellate cells, which not only improved the liver-specific functional properties of PHHs, but also gives us better insights of the liver injury under alcohol treatment. Apart from human hepatic stellate cells, liver consists of Kupffer cells and liver sinusoidal endothelial cells which play a major role in the progression of liver fibrosis and in the healing process. The proposed disease model can be further optimized for other liver cells for a better understanding of the *in vivo* counterpart.

8.2.2 Scanning of drugs and toxins

The present study optimized a multiple ECM modified 3D electrospun scaffold with primary human hepatocytes and hepatic stellate cells to study alcoholic liver injury. It gives a brief demonstration of the use of this cell-scaffold assembly to study alcoholic liver disease *in vitro*. This model can be further useful to study the effect of different drugs in improving liver-specific functions of the alcohol injured human liver cells. Many drugs have been reported by many research groups over the years, such as, Corticosteroids [288], Pentoxifylline [289], Colchicine [290], Propylthiouracil [291], Dilinoleoylphosphatidylcholine [292], S-Adenosylmethionine [293], Vitamin E [294], GSH Prodrugs [295] etc. for treating alcoholic liver diseases. However, only a few

FDA approved drugs are available to treat alcoholic liver diseases (ALD) such as naltrexone, acamprosate, and disulfiram [296]. Thus, extensive research is needed to evaluate other cures for ALD, where this *in vitro* model with human liver cells can be used for the initial screening of such agents. Apart from the ALD study, this proposed model could be helpful to test the cytotoxicity of chemicals and to evaluate the therapeutic windows for antifibrotic drugs. Finally, the proposed model can be further modified and can be subjected to improvisation to test cures for modeling different liver diseases.

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