Enhancement of Hepatocellular Functionality and Cytotoxicity In-vitro by Nanostructure and Chemical Modification of Materials That Mimic In-vivo Environment

体内を模倣した材料のナノパターン形成とその化学修飾による肝細胞機能と毒性応答の向上

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بسم الله الرحمن الرحيم

رَبَّنَا لاَ تُؤَاخِذْنَا إِن نَّسِينَا أَوْ أَخْطَأْنَا رَبَّنَا وَلاَ تَحْمِلْ عَلَيْنَا إِصْراً كَمَا حَمَلْتَهُ عَلَى الَّذِينَ مِن قَبْلِنَا رَبَّنَا وَلاَ تُحَمِّلْنَا مَا لاَ طَاقَةَ لَنَا بِهِ وَاعْفُ عَنَّا وَاغْفِرْ لَنَا وَارْحَمْنَا أَنتَ مَوْلاَنَا فَانْصُرنا

صدق الله العظيم

(القران العظيم, سورة البقرة)

By the name of Allah

"Our Lord, do not impose blame upon us if we have forgotten or erred. Our Lord, and lay not upon us a burden like that which You laid upon those before us. Our Lord, and burden us not with that which we have no ability to bear. And pardon us; and forgive us; and have mercy upon us. You are our protector, so give us victory

(Holy Quran, Surat Albakarah)

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List of abbreviations

Abbreviation	Full terminology
ECM	Extracellular Matrix
RGD	Arginine-Glycine-Aspartic
EBL	Electron beam lithography
ALD	Atomic layer deposition
TOF-SIMS	Time of flight secondary ion mass spectroscopy
XPS	X—ray photoelectron spectroscopy

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Chapter 1

Liver cells functionalities, responses, applications and recent challenges.

1. The role of liver cells in the body and outside it

The liver is considered as the largest internal organs of body, it is mainly composed of parenchyma cells ‘hepatocytes’ then a lesser number of stellar (fat storing cells), kupffer (specific liver macrophage), sinusoidal endothelial cells (Figure 1-1), its essentiality is based upon the metabolic, secretory and detoxifying functions which performed by the hepatocytes. Lipid metabolisms, erythropoises, and protein synthesis are also performed by liver cells [1]. It plays the key role in the metabolism of endogenous and exogenous entities and molecules as drugs, chemicals, and toxins. Because of this secretory, metabolic and detoxifying functionalities, hepatic cells are important for drug development programs.

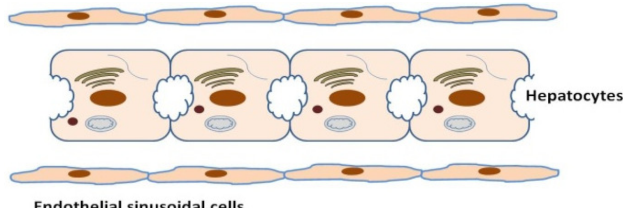

Figure 1-1: Hepatocytes arrangements inside the liver

In pharmaceutical industries, hepatic cells and their culture systems *in-vitro* are widely used for the analysis of novel drugs (Figure 1-2). The prediction of side effects and hepatic biochemical alterations are constantly investigated especially after the exposure of the liver cells to foreign molecules. Furthermore, the determination of metabolic responses and further metabolites is one of important aspects for drug discovery programs outcomes to evaluate its cytotoxicity, since, some drugs have little cytotoxicity, however, in the liver they undergo further metabolism into more toxic molecules [2]. Consequently the expressions of maximized functional proteins and enzymes from the liver cells are the key factors for the

validity of drug testing and analyses. On the contrary, culturing of hepatic cells *in-vitro* is associated with a reduction in the hepatocellular functionalities and decreased secretion of liver specific proteins compared to cells in their native environment [3]. This declined hepatic functionalities resulted lower toxicity associated with *in-vitro* testing and analyses of chemicals such as (clozapine, isoniazide) despite clinically proven to be hepatotoxic [4]. Consequently, the decrease in hepatic cellular functions associated with *in-vitro* culturing technique will represent a challenge for the reproducibility and standardization of its application in drug development and *in-vitro* analysis (Figure 1-3)

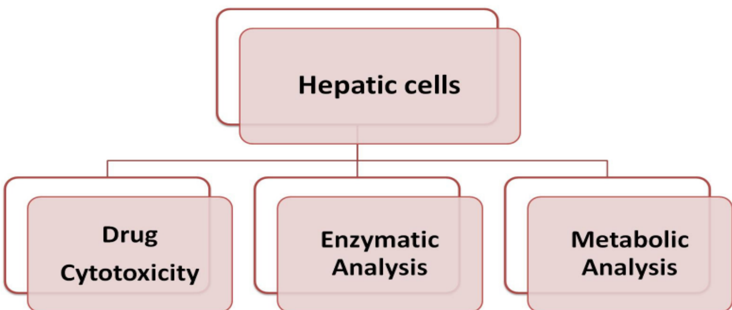

Figure 1-2: Scheme describes the applications of hepatic cells in the pharmaceutical industry and *in-vitro* drug testings.

The alteration in the hepatocellular functionalities *in-vivo* and *in-vitro* are associated with the absence of the liver native cellular environments or extracellular matrix (ECM) especially by the use 2D- flat cell culture techniques. Thus, ECM is a crucial determinant of the compliance and functionality of liver despite its lower overall percentage. It provides the structural frameworks and essential cues to maintain the hepatocytes states and related functionalities at the highest level.

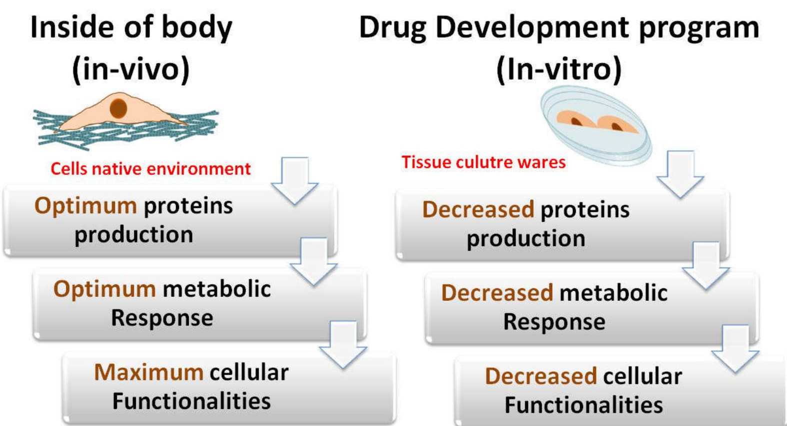

Figure 1-3: Shows the discrepancies in liver cellular behaviorss *in-vivo* & *in-vitro*

2. ECM, compositions, and its functionalities

ECM is the cellular native environment that is composed of complex, well-defined organic matrices that incorporate several macromolecules and proteins. The ECM is assembled into a three-dimensional network containing precisely contoured nanostructures (Figure 1-4). The existence of this intricate mesh works of assembled macromolecules is mainly tissue specific, since mainly compositions, concentrations, and geometries of these macromolecules depend on tissue specific structural demands [5].

ECM macromolecules include various proteins, glycoproteins and proteoglycans. The major non-proteinaceous component of ECM called glycosaminoglycan (GAG) hyaluronan. The most ubiquitous proteins of ECM include collagens, fibronectin, elastin, laminin and fibrillins. These diversified compositions play a leading role in orchestrating cellular behaviors.

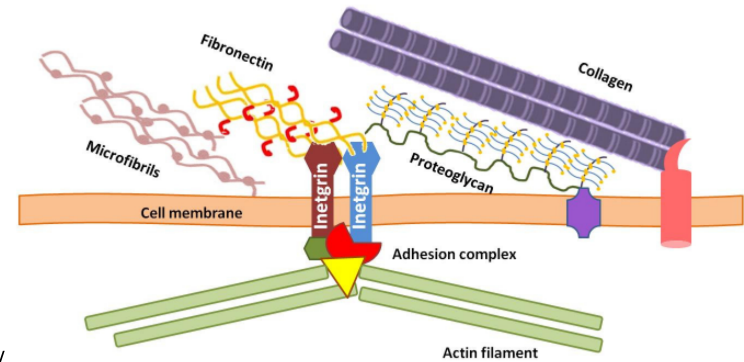

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Figure 1-4: Illustrative cartoons for extracellular matrix intricate meshworks and their interactions with plasma membrane.

In the past, ECM was considered as an inert template to maintain the physical structures of cells. However, this was inaccurate definition for the role of ECM sophisticated environments. Although each cell has its inherited characteristics on its genome, there is a continuous interplay with ECM characteristics. Since a modulation in ECM characteristics leads to modulation of cellular behaviors such as proliferation [6], adhesion [7], migrations [8], differentiation [9], development [10]. For the performance of this cell instructive roles, the ECM assembly and composition are not constant, it is more likely in continuous variations depends on the cellular demands, this done by the intermolecular cross-linking or proteinase with the cellular secretions of posttranslational enzymes as matrix metalloproteinases (MMPs).

2.1 Various ECM cues and its role.

The word "*Cue*" defines anything that could stimulate an action. The term ECM cues usually describe the multi-structural and functional entities located in extracellular matrix and native environment around cells to control their behaviors. This distinctive cues

stimulate cell specific response and tissue compliance in different organs. Furthermore, they have a central and fundament role in embryogenesis and development. These cues is divide into 2 types; physical and chemical cues as will be discussed.

The ECM provides the mechanical strength and support for the cells by acting as architectural frameworks. Such frameworks are resulted from the assembly of various ECM components as previously mentioned. The constructions of these frameworks appear in the form of several prominences, pores, fibers and even random projections/depressions which located in the nano and micron size. The presence of three dimensional topographies is an important reason for the control of cellular performance [11]. Not only have the geometries of such ECM biophysical features but also their size correlated with specific tissue functionality.

The configurations and compositions of ECM frameworks resulted likewise further modifications in matrix stiffness as well as surface roughness that in turn alter the overall cell behaviors [12]. Such variable mechanical and architectural characteristics of ECM are called physical cues. These cues can be considered as intrinsic regulators of cellular behaviors as they act mechanotrasduction forces to alter the cellular characteristics and behaviors in different tissues. For instance, ECM of human cells mainly composed of collagen (most abundant ECM protein), but diversified functionalities and tissue compliance could be relatively attributed to the significant architectural biophysical difference between tissues in addition to other factors [13].

Since the ECM composed of various kind macromolecules with diversified functional groups and chemical divergence, The variations in them have resulted in subsequent interactions with cell surface receptors and ligands consequently triggering intracellular signaling cascades to manipulate the cellular responses. Furthermore, acting as a reservoir

for chemokines or cytokines and growth factors has been associated with alterations in cellular hemostasis. Such biochemical instructive tissue specific characteristics of ECM can describe the term of ECM chemical cues with distinctive cell manipulative properties.

3. Mimicking Liver microenvironment and ECM cues for the improvement of hepatic behaviors

In the last two decades, diversified techniques (natural or artificial) were developed to mimic the unique ECM cues of liver microenvironments. The development of techniques plays a key role in drug testing strategies and bioartificial liver related applications. The most renowned techniques to mimic the liver ECM was illustrated in Table 1-1. Despite the diversified techniques for liver-ECM simulation and even their further use simultaneously to maintain the innate hepatic functionalities, hepatocellular culture *in-vitro* with a high cellular functionalities is still a challenge especially in the biomaterial fabrication for *in-vitro* drug development strategies and related applications. Accordingly, an understanding of the relationship between hepatic cells and their environmental native cues is essential for the cell responsive biomaterial fabrication technology.

Table 1-1: The characteristics and several methodologies for mimicking hepatic microenvironments and various ECM cues.

Method	**Characteristics**	**Ref.**
2D tissue culture plate covered with ECM proteins as collagen, fibronectin	- Inexpensive, reproducible and robust. -Lower hepatic functionalities as insufficient mimic of 3D liver microenvironment. Short-lived canalicular structures. -Varied responses depending on type of protein used.	[14, 15]
ECM Protein sandwich configurations for hepatocytes culture	-*In-vivo* like morphology. -Inaccurate nutrients transportation. -Mechanical instability.	[16]
Coculture system (another type of cells with hepatocytes) Single or double layer	-Production of native ECM and growth factors by such adjutant cells. -Low mechanical stability. -Responses are adjutant cell type dependant as limitless cell to cell interactions mechanisms.	[17]
Matrigel	Gelatinous proteins mixture produced *in-vitro* from E–H–S mouse sarcoma. High possibility of biological contaminations. Immunogenicity and pathogenic tumor origin.	[18]
Hollow fibers (i.e. alginate)	Hepatocytes are loaded inside or outside hollow fibers. Various polymers with versatile characteristics can be used. Proper oxygen and nutrient transfer is required. Further improvement of hepatocytes functionalities.	[19]
Porous 3D Scaffold	Efficiency mainly depends on the nature of material used. Pore size and dimension are crucial for the proper nutrient and oxygen diffusions.	[20]
Natural Polymer films (i.e. Chitosan)	Lower mechanical integrity. Source variabilities for polymers with natural origin.	[21]
Synthetic polymer (i.e. Polyesters, PEG)	Overcome the lack of cell adhesion motifs by the superficial modifications with biomolecules as RGD. Mutable characteristics for improvement of hepatocellular functions. Diversified models and forms as hydrogels and foams.	[22, 23]
Inorganic materials (i.e.TiO_2, Al_2O_3)	Versatile characteristics & expanded fabrication technologies.	[24,25]

4. Synthetic biomaterials to control hepatocellular responses by mimicking the in-vivo ECM.

The surpassing advancement in the fabrication of synthetic biomaterials with specific characteristics and distinctive structures is one of key reasons for the prevalent utilization of such biomaterials in the liver *in-vitro* applications especially to control the hepatocellular responses in drug development programs. Synthetic materials don't experience immunogenicity, biological contaminants, or origin variability associated with natural based biomaterials [26]. The precise control of structures in nano and micro-scale levels with novel mechanical properties of substrates leads to the improvement and validations of hepatocellular functionalities for the bio-related applications such as drug testing strategies. Moreover, mimicking of various ECM physical cues (i.e. stiffness, topography and roughness) could be easily achieved by the precise construction of such nano and micro-scale entities with controlled morphologies, compositions, shapes and size.

Furthermore, the presence of versatile techniques for the functionalization and derivatization allows mimicking the several ECM chemical cues with distinctive surface chemical functionalities for the realizations of interplays and factors required for the enhanced cell integrations properties [27].

Although ECM is sophisticated structures with multifunctional characteristics, the mimicking of single or double cues using the advanced nanotechnologies is essential for the understanding of the relationship between liver human cells and ECM cues. Thus, the recognition and mimicking of such cues by the alteration in interfacial characteristics of biomaterials using available advanced fabrication techniques would provide a novel mean for the increase of hepatic functionalities especially for *in-vitro* applications.

4.1. Material superficial topography to control the hepatocellular behaviors.

The importance of topography manipulation of substrates (starting from submillimiter to micrometer) for the maximization of hepatocellular functionalities have been a central subject for many researches especially to overcome the decreased hepatic functionalities associated with the use of ordinary flat tissue culture wares and associated challenges for their utilization in valid drug *in-vitro* analyses. Since morphology of superficial features and dimension would influence the hepatocellular adhesion, cytoarchitectures and subsequent functionalities. TiO_2 scaffolds with hundreds micrometer cavities influenced the hepatocytes distributions and adhesions [28]. U-shaped trenches topography with several micrometer size (width 17-30 µm, depth 1-4 µm) promoted the three-dimensional growth of hepatocytes with a subsequent maintenance of higher albumin expression [29]. The microporosity is important determinant of hepatocellular behaviors, since the higher pore size (67µm) of PGLA (DL-glycolic-co-lactic acid) substrates, the higher 3D organizations with a subsequent increase in albumin secretions [30].

. Despite the expected significance of submicro/nano-scale topography in the promotion of hepatic functionalities, they are seldom studied, except the (equally spaced) 800 nm pitches that previously reported to increase the albumin expression based on the alteration of groove depth [31]. This urged me to consider the role of such nano-scale variations in the shape and size of topographical features and the subsequent promotion of hepatocellular behaviors including the expression of several functional proteins. Since the influence of nanofeatures geometrical manipulations may result a variation in cellular morphology, integrins clustering arrangement and cellular alignment, with a subsequent alteration in cellular adhesion and functionalities. Furthermore, I focused on the role of unequal alterations in the dimensional magnitude of topographical features or the heterotopic modifications in the interspaces

between nanofeatures, on which these alterations in interspace dimension can provide the required substratum area for enhancement of integrin interactions and even a smaller interspace can be used for the entrapment of bioactive molecules as ECM proteins with a resultant increase in integrins substratum interactions [32] with expected alterations in cellular cytoarchitectures and functionalities. Consequently, the results of this study would provide the required information for the best size and geometry of nanofeatures that would enhance multiple hepatic functionalities. This information can be further exploited for the fabrications of biomaterial substrates that could be utilized in hepatocellular *in-vitro* culture wares for more sensitive drug cytotoxicity analyses (chapter 2, 4).

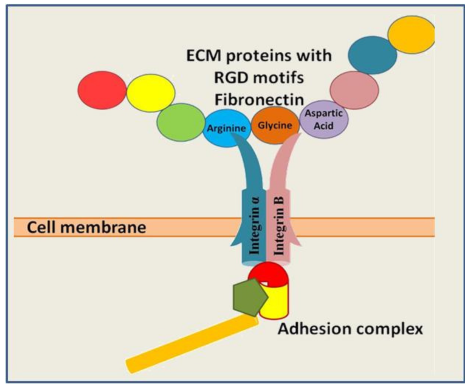


Figure 1-5: shows presence of RGD motif in ECM protein "fibronectin and its interactions with integrins

4.2. Chemical functionalization of the biomaterial using RGD to control the hepatocellular behaviors.

RGD motif (Arginine-Glycine-Aspartic) is tripeptide sequence that is considered as a cell binding domain in many ECM proteins as fibronectin. RGD is responsible for the regulation of many cellular functions as fibronectin assembly, cellular adhesion through their interactions with integrins receptors (i.e. $\alpha5\beta1$) (Figure 1-5). Integrins are heterodimeric (α, β

subunit) transmembrane receptors that transduce the signals arise from the external environment as ECM into cells through triggering cascade of cellular signaling pathways. It is essential to further mediates cellular adhesion, since a site directed mutagenesis in RGD motif with the replacement of glutamic acid for aspartic acid resulted loss of the cell adhesive capability [33].

The associations of peptide sequences in various biomaterial surfaces have been reported to increase the expression of hepatocellular proteins and biomarkers such as albumin, cytochrome P-450 [34, 35]. These enhanced functionalities can be explained due to the increase in the percentage of attached cells; however, this may not be the case of RGD-hepatocellular interactions. Since it was previously reported the hepatocytes functionalities is altered upon the immobilization of 2 forms of RGD sequences (one is RGD in a short peptide sequences, other is a bigger peptide with RGD sequences), although the hepatocellular percent of attachment between 2 RGD coated substrates is the same [36]. Such change in the hepatocytes functionalities were attributed to RGD-cell receptor conformations, interactions and affinities with subsequent change in the cellular morphology yet not attachment. Because of this change in the RGD-receptor interactions, an alteration in cellular architectures would be observed with subsequent change in sophisticated biochemical signaling cascades and resultant functionalities. The roles of RGD sequences or proteins that contain such motif in the regulation of hepatocellular functionalities have been previously reported [34-36] however, in the nature, the role of RGD as an important regulator of liver functionalities is not isolated from other biochemical factors and physical cues of ECM. Consequently, it may be significant to investigate the impact of combining more than a single cue in the same substrate to closely mimic the natural liver environment. So the role of integration of RGD with the physical manipulation of topographical characteristics of

materials and the hepatic responses to such integrations were studied and compared to substrates with each cues alone or substrate without any cue (chapter 3).

5. Motivations

The design and fabrication of cell culture models with appropriate chemical compositions, and biophysical characteristics and assemblies are still problematic especially for the promotion of hepatic functionalities. These models would be utilized as cell culture substrates in the developments of validated *in-vitro* drug analyses strategies as the measurements of cytotoxicity. Despite the presence of several techniques to mimic the bulk hepatocellular native environments (ECM), the relationship between single or few ECM elemental cues and hepatic cellular behaviours especially at the nano-level needs to be investigated. This relationship will enable us to anticipate the best essential superficial characteristics for the increased cell-substratum interactions. Furthermore, In drug analysis strategies, the utilization of primary hepatocytes (directly isolated from liver) is associated with several variabilities, fast loss of functionalities, cost limitations, while the utilization of other types of immortalized hepatic cells (cancer cell lines) in drug development applications will result insignificant and inaccurate analyses results due to their decreased functionalities despite other advantages as low cost and easy handling. Thus, Here, Our purpose was the design and fabrications of substrates that enhance the hepatic functionalities for more sensitive cytotoxic drug analysis. HepG2 was chosen as a testing hepatic model for the investigation of such substrate superficial characteristics. It is a hepatic cancer cell line with low hepatic functionalities *in-vitro*. It is stable cells compared to primary hepatocytes that experience morphological and genetic alterations owing to the abrupt loss of their native environments after isolations. It is characterized by low cost and easy handling which fits in the pharmaceutical industrial drug analyses.

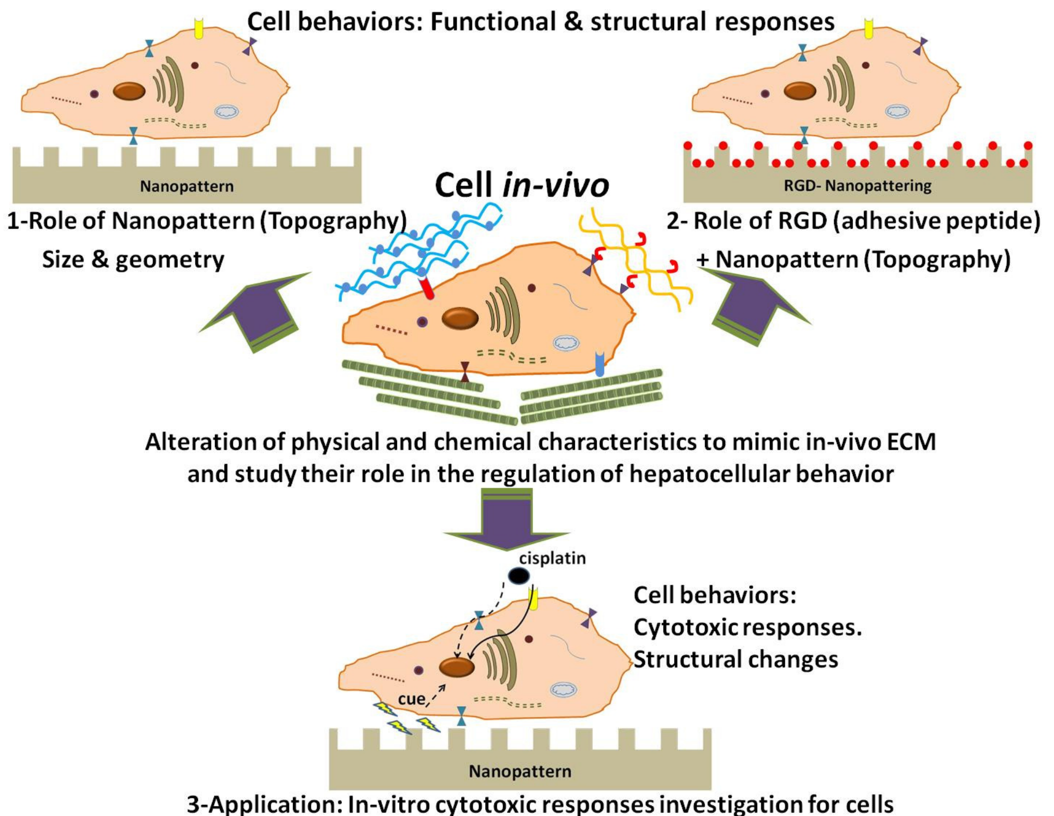


Figure 1-6: Depicts the 3 strategies used to examine the role of chemical and physical variations in nanopattern to mimic ECM cues and the tested hepatocellular *in-vitro* responses.

6. Hypothesis and strategies to test such hypothesis

In order to achieve this purpose, I have hypothesized that the variations in the chemical and physical characteristics of substrates specifically to mimic ECM cues using the available advancement in nanofabrication techniques can be used to improve the hepatocellular functionalities and behaviors. Thus, this manipulated surfaces can be utilized for more sensitive *in-vitro* drug analyses and cytotoxicity studies.

3 steps were done to test my hypothesis, at the beginning; mimicking of the single/multiple ECM cues for the expression of cell responsive surface followed by the investigation of *in-vitro* hepatocellular behaviors in order to promote and alter their functional and structural responses were done. At the end, the utilization of such cell instructive substrate as a culture system was done for *in-vitro* analysis of drug cytotoxicity "Cisplatin". Three strategies were adapted to test this hypothesis and they are illustrated in figure 1-6 and explained in details in chapters 2-4.

1) Mimicking ECM physical cues (Topography) by the change in size and geometry of nanostructures

I- Design of cell responsive topographical cues for the promotion of hepatocellular behaviors

In order to achieve such a target, 3 major factors should be taken into considerations.

-Individual ECM cues (shape, size) and optimum dimensions.

-Cellular identities and their *in-vivo* architectures

- Desired cellular responses.

Thus, when I choose hepatic cells as a candidate for such hypothesis, I tried to mimic the geometry and size of the most ubiquitous ECM proteins "collagen" in liver. The elongated linear geometry was chosen to mimic the hierarchically extended collagen fibrils. Then the importance of parallel configuration was tested by the use of another geometry "discontinuous rectangular" with the same dimension (Figure 1-7). the native collagen fibrils with (60 nm) diameters are subsequently arranged into bundles with larger diameters (0.2-5µm) [37]. For mimicking the optimum dimension for hepatic responese, 3 dimensional characteristics involve, diameter of nanostructures or gratings (X), its height (Z), and the interspace in between such diameters (Y) should be chosen. The diameters of the

nanogratings (X) were chosen to resemble the collagen fibrils sizes. The height (Z) we chose as 40 nm, since a larger height will confine the attachment of the cells over the nanogratings without any further cellular access to the interspace between nanogratings with resultant constrictions of integrins assemblies on the upper features. Finally, heterotropic alteration in the diameter/interspace (X:Y) dimensions to fit within ≃ 1:2, 1:3, and 1:4 ranges.

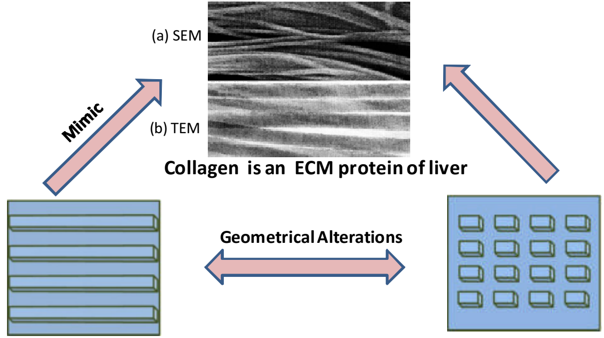


Figure 1-7: Depicts the resemblance between fabricated nanogratings and one of liver ECM proteins "collagen" and the geometrical alterations that performed into another shape, SEM and TEM for ECM collagen of liver obtained from published article [37].

II- Fabrication of nanopattern with such specific dimensional and geometrical characteristics and surface coating with TiO_2 film.

Electron beam lithography was used to perform the nano-scale physical and topographical variations in the surface of silicon wafers as illustrated in chapter 2 in details. Pristine silicon wafers may not be the bio-efficient environment since fluctuations in affinities and decreased cellular adhesion can be observed dependent on cell types [38]. While the adhesions and attachments of cells over the biomaterials are essential determinants of cell-substratum interactions and subsequent resultant cellular responses. Consequently, surface coatings of such nanostructures with a thin film of metal oxides or organic polymeric materials were our option. TiO_2 was chosen as a scaffold for my models to increase the biocompatibility and bioactivity of my substrates. I cannot conclude the main role of TiO_2

since I have not compared it with other surface coatings. However, I believe that presence of such films will increase the biocompatibility, since TiO_2 thin films have previously reported to increase the proteins adsorption especially serum proteins consequently favors cell material interactions with a resultant increase in cellular adhesion [39]. TiO_2/chitosan composites had a higher attachment efficiency with a higher expression of hepatic marker "albumin" compared to chitosan alone [40]. The deposition of thin film of TiO_2 using atomic layer deposition technique was able to increase the protein adsorption and impart hemostatic characteristics to cellulose acetate fibers [41]. Furthermore, TiO_2 thin films as a scaffold were able to induce the expression of various membrane receptors and signaling proteins involved in cell-substratum interactions as integrin β1, and activations of ERK1 and ERK-2 with enhancement of cellular attachment and subsequent functionalities [42]. Accordingly, the thin films of TiO_2 could facilitate the cell material interactions with subsequent binding of specific biological targets as proteins, cellular receptors. Additionally, the unique intrinsic features of TiO_2 as surface characteristics as thermal stability and antibacterial properties that increase its utilization in biomaterial fabrications [43]. Thus, TiO_2 structural characteristics (the size, morphology, topography, wettability, crystallinity) provide the essential aspects of cell-environment interactions with subsequent alteration in cell organization, survival, and functions in various cell types as chondrocytes [44], osteoblast [45], endothelial cells [46], mesenchymal stem cells [47]. This particularly true for liver cells on which flat TiO_2 films increased the cell-cell interactions that enabled the arrangement for hepatocytes in 3D structures after several days with a subsequent increase in hepatic functionalities [25]. We tried to avoid the TiO_2 associated disadvantage as photoactivity with the emission of reactive oxygen species upon exposure to light with certain wavelength by the use of amorphous TiO_2 with the minimum photocatalytic activity.

III- Asses the impact of variable geometrical and dimensional cues in substrates characteristics on the hepatic functionalities. This was done by investigating the expression of hepatic proteins (i.e. albumin, transferrin, and cytochrome P-450).

IV- Characterize the structural cellular response for change in the geometry of nanostructures (alignment, adhesions, ECM assembly).

2- Simultaneous mimicking ECM physical (Topography) and chemical (RGD) cues in a single substrate.

The critical role of physical and chemical cues collectively and separately on the hepatocellular behavior was determined.

I- Immobilization of RGD as an adhesive peptide on the surface of pattern and flat substrates.

II- Evaluation of hepatocellular functionalities to determine the importance of ECM cues integration in the hepatic protein expressions.

III- Characterize the structural cellular response for single/integrated cues (i.e. alignment, adhesions, ECM assembly).

3-An application: *In-vitro* cytotoxic analysis of cisplatin using nanopattern substrates as a culture system.

For the achievement of more sensitive *in-vitro* drug analyses: the hepatocellular responses to a cytotoxic agent 'cisplatin were examined while cells cultured on substrates with a modified topography that mimics ECM physical cues. The cellular changes were compared due to the presence of such nanostrcutures after the exposure to a cytotoxic agent.

I- Determination of cell-substratum induced variations and cisplatin cytotoxic response.

II- Characterize the structural cellular response to the physical cues under a cytotoxic platform (i.e. chromatin condensation, alignment, morphology).

The first and second strategies were implemented to determine the specific physical and chemical variations essential for the promotions of hepatocellular behaviors in order to determine the required tangible superficial characteristics of biomaterial with subsequent increase in hepatic functionalities. After that, the last strategy was implemented to investigate the utilization of such superficial characteristics (Topography) for more sensitive *in-vitro* drug testings. Topographical alterations in nanostructures and its influence on hepatocellular structures and functionalities are illustrated in chapter 2. While the simultaneously combining physical and chemical variations in a single substrate and its influence on hepatocellular responses were examined in chapter 3. After that we tried to validate our results by examining such substrate as a culture system for *in-vitro* drug toxicity analysis as illustrated in chapter 4. Finally we can conclude that the specific recognition and mimic of ECM physical and chemical cues separately or simultaneously could remarkably used to attain the innate hepatic functionalities *in-vitro* specifically for drug analysis applications as illustrated in the conclusion remarks.

7. References

[1] Vasanthan KS, Subramanian A, Krishnan UM, Sethuraman S, Role of biomaterials, therapeutic molecules and cells for hepatic tissue engineering, Biotechnol., Advances. 2012; 30: 742‑752.

[2] James LP, Mayeux PR, Hinson JA, Acetaminophen-induced hepatotoxicity, Drug Met., Deposition, 2003; 31: 1499-1506.

[3] Martinez-Hernandez A, Amenta PS, The hepatic exctracellular matrix. I components and distribution in normal liver. Virchows Archiv A pathol Anat., 1993; 423: 1-11.

[4] Menge Q, Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity, Expert Opin Drug Metab Toxicol., 2010; 6: 733-746.

[5] Hubmacher D, Apte SS. The biology of the extracellular matrix: novel insights, Curr Opin Rheumatol., 2013; 25: 65–70.

[6] Raines EW, The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. Int J Exp Path., 2000; 81:173–182.

[7] Kim S-H, Turnbull J, Guimond S, Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol., 2011; 209: 139–151.

[8] Wolf K, Friedl P, Extracellular matrix determinants of proteolytic and non-proteolytic cell migration, Trends Cell Bio., 2011; 21: 736-744

[9] Watt FM, Huck WTS, role of extracellular matrix in regulating the stem cell fate, Nat Rev Mol Bio., 2013; 14: 467-473.

[10] Rozario T, DeSimone DW,The extracellular matrix in development and morphogenesis: A dynamic view, Develop Biol., 2010, 341: 126‑140.

[11] Liliensiek SJ, Nealey P, Murphy CJ, Characterization of Endothelial Basement

Membrane Nanotopography in Rhesus Macaque as a Guide for Vessel Tissue Engineering, Tissue Eng: Part A., 2009; 15: 2643-2651.

[12] Wells RG, The role of matrix stiffness in regulating cell behavior, Hepatology, 2008; 4:1394-1400.

[13] Gasiorowski JZ, Murphy CJ, and Nealey PF. Biophysical Cues and Cell Behavior: The Big Impact of Little Things, Ann Rev Biomed Eng., 2013; 15; 155-176.

[14] Knop E, Bader A, Boker K, et al. Ultrastructural and functional differentiation of hepatocytes under longterm culture conditions, Anat Rec., 1995;242:337-49

[15] Schuetz JD, Schuetz EG, Extracellular Matrix Regulation of Multidrug Resistance in Primary monolayer cultures of adult rat hepatocytes, cell growth Diff., 1993; 4: 31-40.

[16] Berthiaume F, Moghe PV, Toner M, Yarmush ML, Effect of extracellular matrix topology on cell structure, function, and physiological responsiveness: hepatocytes cultured in a sandwich configuration, FASEB J., 1996; 10: 1471-1481.

[17] Bhandari RNB, Riccalton LA, Lewis AL, Fry JR, Hammond AH, Tendler SJB, Shakesheff KM, Liver Tissue Engineering: A Role for Co-culture Systems in Modifying Hepatocyte Function and Viability, Tissue Eng., 2001; 7: 334-357.

[18] Page JL,; Johnson MC,; Olsavsky KM, Strom SC, Zarbl H, Omiecinski CJ, Gene expression profiling of extracellular matrix as an effector of human hepatocyte phenotype in primary cell culture, Toxicol Sci., 2007; 97: 384–397.

[19] Palakkan AZ, Raj DK, Rojan J, Sajin Raj RG, Anil Kumar PR, Muraleedharan CV, and Kumary TV, Evaluation of Polypropylene Hollow-Fiber Prototype Bioreactor for Bioartificial Liver, Tissue Eng: Part A., 2013; 19: 1056-1066.

[20] Dvir-Ginzberg M, Gamlieli-Bonshtein I, Agbaria R, Cohen S, Liver Tissue Engineering within Alginate Scaffolds: Effects of Cell-Seeding Density on Hepatocyte Viability,

Morphology, and Function, Tissue Eng: Part A., May 2003; 9: 757-766.

[21] Elçin M, Dixit V, Gitnic G, Hepatocyte Attachment on Biodegradable Modified Chitosan Membranes: *In-vitro* Evaluation for the Development of Liver Organoids, Artificial Organs, 1998; 22: 837-846.

[22] Park KH, Bae YH, Phenotype of hepatocyte spheroid in Arg-Gly-Asp (RGD) containing a Thermo-Reversible extracellular matrix, Biosci Biotechnol Biochem., 2002; 66: 1473–1478.

[23] Iijima H, Nakazawa K, Mizumoto H, Matsushita T, Funatsu K. Formation of a spherical multicellular aggregate (spheroid) of animal cells in the pores of polyurethane foam as a cell culture substratum and its application to a hybrid artificial liver, J Biomater Sci Poly., 1998; 9: 765–78.

[24] Hoess A, Teuscher N, Thormann A, Aurich H, Heilmann A, Cultivation of hepatoma cell line HepG2 on nanoporous aluminum oxide membranes, Acta Biomater., 2007; 3: 43-50.

[25] Nakazawa K, Lee S-W, Fukuda J, Yang D-H, Kunitake T, Hepatocyte spheroid formation on a titanium dioxide gel surface and hepatocyte long-term culture, J Mater Sci: Mater Med., 2006; 17: 359-364.

[26] Vasanthan KS, Subramanian A, Krishnan UM, Sethuraman S, Role of biomaterials, therapeutic molecules and cells for hepatic tissue engineering, Biotechnol Adv., 2012; 30: 742–752

[27] Petronis S, Eckerl K-L, Gold J, Wintermantel E, microstructuring ceramic scaffolds for hepatocytes cell cultures, J Mater Sci: Mater Med., 2001; 12: 523-528.

[28] Mandes PM, cellular nanotechnology mimicking biological interfaces and smarter, Chem Soc Rev., 2013; 42: 9207-9218.

[29] Kaihara S, Borenstein J, Koka R, Lalan S, Ochoa ER, Ravens M, Pien H, Cunningham B, Vacanti JP, Silicon micromachining to tissue engineer branched vascular channels for liver fabrication, Tissue Eng., 2000; 6:105-17.

[30] Ranucci CS, Moghe PV, Polymer Substrate Topography Actively Regulates the Multicellular Organization and Liver-Specific Functions of Cultured Hepatocytes, Tissue Eng., 1999; 5: 407-420.

[31] Tsai W-B, Lin J-H, Modulation of morphology and functions of human hepatoblastoma cells by nano-grooved substrata, Acta Biomater., 2009; 5: 1442-1454.

[32] Biggs MJP, Ritchard RG, Dalby MJ, Nanotopographical modification: a regulator of cellular function through focal adhesions, Nanomed: Nanotechnol Biol Med., 2010; 6: 619-633

[33] Obara M, Kang MS, Yamada KM, Site-directed mutagenesis of the cell binding domain of human fibronectin separable synergistic sites mediate adhesive function, Cell. 1988; 55: 649-657.

[34] Bartolo LD, Morelli S, Lopez LC, Giorno L, Campana C, Salerno S, Rende M, Favia P, Detomaso L, Gristina R, d’Agostino R, Drioli E,Biotransformation and liver-specific functions of human hepatocytes in culture on RGD-immobilized plasma-processed membranes. Biomaterials, 2005; 26: 4432-4441.

[35] Du Y, Chia S-M, Han R, Chang S, Tang H, Yu H, 3D hepatocyte monolayer on hybrid RGD/galactose substratum, Biomaterials, 2006; 27: 5669-5680.

[36] Bhadriraj K, Hansen LK, Hepatocyte adhesion, growth and di!erentiated function on RGD-containing proteins, Biomaterials, 2000; 21: 267-272.

[37] Ohtani O, Three-dimensional organization of the collagen fibrillar framework of the human and rat livers, Arch Histol Cytol., 1988; 51: 473–488.

[38] Corey JM, Brunette AL, Chen MS, Weyhenmeyer JA, Brewer GJ, Wheeler BC. Differentiated B104 neuroblastoma cells are a high resolution assay for micropatterned substrates, J Neurosci Methods, 1997;75:91-97.

[39] S. R. Sousa SR, Moradas-Ferreira P,Saramago B, Viseu Melo L, Barbosa MA, Human Serum Albumin Adsorption on TiO_2 from Single Protein Solutions and from Plasma, Langmuir, 2004; 20: 9745-9754.

[40] Zhao L, Chang J, Zhai W, Preparation and HL-7702 cell functionality of titania/chitosan composite scaffolds, J Mater Sci: Mater Med., 2009; 20: 949–957

[41] Hyde GK, Stewart SM, Scare G, Parsons GN, Shih C-C, Shih C-M, Lin S-J, Su Y-Y, Monteiro-Riviere NA, Narayan RJ, Atomic layer deposition of titanium dioxide on cellulose acetate for enhanced hemostasis, Biotechnol J., 2011; 6: 213–223.

[42] Zhang S, Sun J, Xu Y, Qian S, Wang B, Liu F, Liu X, Biological Behavior of Osteoblast-like Cells on Titania and Zirconia Films Deposited by Cathodic Arc Deposition. Biointerphases, 2012; 7:1-10

[43] Tan AW, Pingguan-Murphy B, Ahmad R, Akbar SA, Advances in fabrication of TiO_2 nanofiber/nanowire arrays toward the cellular response in biomedical implantations: a review, J Mater Sci., 2013; 48:8337–8353

[44] Burns K, Yao C, Webster TJ, Increased chondrocyte adhesion on nanotubular anodized titanium, J Biomed Mater Res Part A., 2009; 88:561-568.

[45] Oh S, Daraio C, Chen LH, Pisanic TR, Finones RR, Jin S, Significantly accelerated osteoblast cell growth on aligned TiO_2 nanotubes, J Biomed Mater Res A., 2006; 78:97-103.

[46] Mohan GC, Sreerekha PR, Divyarani VV, Nair S, Chennazhi K, Menon D, Influence of titania nanotopography on human vascular cell functionality and its proliferation *in-vitro*, J. Mater. Chem., 2012, 22, 1326-1340.

[47] You M-H, Kwak MK, Kim D-H, Kim K, Levchenko A, Kim D-W, Suh K-Y, Synergistically Enhanced Osteogenic Differentiation of Human Mesenchymal Stem Cells by Culture on Nanostructured Surfaces with Induction Media, Biomacromolecules, 2010; 11: 1856-1862.

Chapter 2

Manipulation of physical characteristics of nanostrcutures that mimic the ECM physical cue to control hepatocellular responses.

1. Summary

The role of bio-inspired superficial topography on the cellular behavior of a hepatocyte cell line was investigated. Nanopatterns with diversified shapes (nanogratings/nanorectangles) and heterotropic lateral dimensions were fabricated using electron beam lithography and atomic layer deposition. These topographical nanocues resulted in the control and regulation of multiple hepatocellular functions. Twelve hours after cell culturing, nanogratings with a lateral dimension of 240 nm showed a higher degree of functional protein expression compared to other nanotopographical substrates and a flat surface. These findings suggest that the nanogratings which resemble a hierarchically extended collagen nanofibrillars could be recognized by hepatic cell line that would resulted in mimicking the in-vivo cytoskeletal orientation and cellular integrity. Therefore, nanopattern with a specific shape and dimension (90nm gratings, 150nm interspace) might emulate collagen fibrils or the intrinsic topography of ECM to enhanced cellular functionalities These unique surfaces could be further exploited for tissue engineering and bioreactor technology.

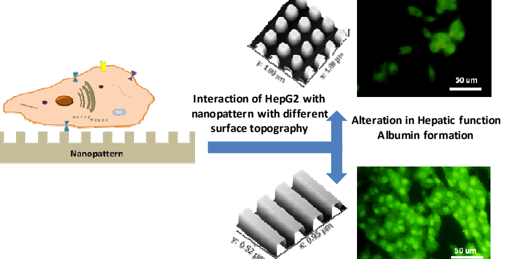


Figure 2-1: Shows the interaction between various nanopatterns that mimic of ECM cues and the resulted alteration in the hepatocellular function “Albumin secretion.

2. Background

The interaction between human cells and biomaterials has been an intense area of study in the last several decades especially when these cellular interactions are cell- and material specific since they are affected by the characteristics of materials as topography, stiffness, surface chemistry. These studies have provided promising approaches for the production novel biomaterials with enhanced cell integration properties, [1-3] and have participated in the production of materials with implantable and bioartificial characteristics at both the research and clinical level.

Living cells in their native environment are embedded in complex, well-defined organic matrices that incorporate macromolecules to provide the extracellular matrix (ECM). The ECM is assembled into three-dimensional meshworks containing precisely contoured nanostructures. The architecture of the ECM possesses the essential physical cues and biochemical factors that trigger and control specific reactions and cellular behaviors [4]. Collagen is one of the ECM molecules. It is a fibrous protein approximately 300 nm long and 1.5 nm wide with a triple helical structure [5]. These helices are assembled into nanofibrillar networks that hierarchically extend for tens of micrometers in length and have diameters of between 30 and 410 nm [6]. It is believed that the micro/nano-texturing features of ECM provide the mechanotransductive cues that deeply influence cell morphology [7], migration [8], proliferation [9], and cytoskeleton organization [10]. Consequently, manipulation of biomaterial superficial texturing and topographical features by the huge advancement in nanofabrication techniques would result an alteration in the cellular functions by influencing the physical cues that trigger various biological responses [11]. Electron beam lithography (EBL) is one of widely used technique for fabrication of small nanostructures that closely mimic the native ECM architectures by sculpting various

nanostructures with diversified nano-meter dimensions and geometries to obtain patterns with specific ECM topographical characteristics [12].

Liver cells need a distinctive natural environment to maintain high levels of cellular function. The cellular function of hepatic cell lines usually decreases when cultured on two-dimensional cell culture surfaces. Thus, the mimicking of native ECM physical and chemical cues to simulate the *in-vivo* cellular architecture (avoid flat architecture with 3D cubic configuration) and maintain innate cellular function is one of suggested reasons to overcome their lowered functionalities. Several techniques have been designed to mimic the innate ECM of liver cells, such as the use of co-culture systems [13], polymers [14], recombinant proteins [15] and inorganic materials [16]. In particular, the deposition of an ultrathin layer of inorganic material such as amorphous titanium oxide which results an increase in the hepatic cell-cell interactions with increased expression of functional proteins as albumin [17]. TiO_2 thin films have the advantage of controllable thickness, as well as facile surface modelling by the simple incorporation of different organic moieties into the deposited inorganic film. Furthermore, modification or manipulation of the surface characteristics and topographical features of TiO_2 based nanomaterials have been shown to change the biological behavior of various cells *in-vivo* and *in-vitro* [18, 19].

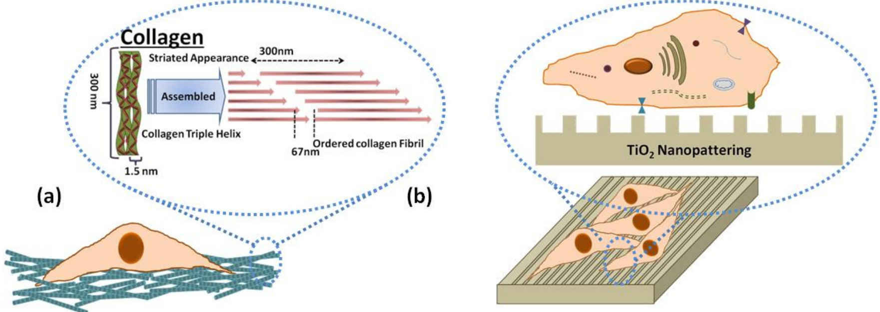


Figure 2-2: Schematic depicts the resemblance between the interactions of Human cells with (a) natural ECM component collagen with its striated appearances, and (b) nanopatterns.

Most previous research has focused on the anisotropic nanotopography with equally spaced dimensions, without considering the impact of unequal alterations in the dimensional magnitude of topographical features on cellular functions [20-22]. While a very few researches focused on the unequal alterations in nanostructures sizes and interspaces between them even though the heterotropic variations in size and interspace have previously reported to induce better early bone response *in-vivo* compared to anisotropic dimension of the same total dimension [23]. These heterotropic variations could alter intergrin clustering, adhesion and subsequent cell-substratum interactions by the realization of the various integrins binding sites in between nanofeatures. Furthermore, the significance of different shapes and models of nanotopographical features based upon their abilities to induce a change in the cellular morphology, alignment, and integrins mediated adhesion with a further evoke of biochemical signalling pathways and functionalities. We investigated the manipulation of nanostructures geometry and interspace size of nanopatterns in nano and submicron scale in order to mimic the dimensional and geometrical characteristics of the fibril structure of collagen. This manipulation was performed using electron beam lithography and atomic layer deposition. The diameter of the nanostructures and interspaces were chosen to resemble the dimensions of hierarchically extended collagen nanofiber networks. The morphological and functional changes in the hepatocytes induced by the diversified nanofeatures were investigated; for example, the alignment and secretion of different functional proteins was observed using fluorescent immunostaining techniques. Our goal was to better understand superficial characteristics such as the optimum size and geometry of tangible nanocues. In order to develop a model that mimics the unique features of ECM. Consequently, we investigate the functional performance of a liver cell line cultured in various models *in-vitro* by observing the expression of various hepatic functional proteins. These cell culture models

could be further exploited in future research and clinical studies.

3. Results

In this study we investigated the fabrication of various nanopattern substrates with specific dimensional and geometrical characteristics to stimulate specific hepatocellular functions. These fabricated constructs mimic the native environment around cells.

Table 2-1: Various 3D figures from AFM analysis of fabricated dimensions and shapes of TiO_2 coated nanostructures (X) = 120, 90 nm, (Y) = 150, 160, 220, 320 nm.

Feature Shape	Code	X (nm)	Y (nm)	X+Y (nm)	AFM
Nanogratings	(A)	90	150	240	
	(B)	120	160	280	
	(C)	90	220	310	
	(D)	90	320	410	
Nanorectangles	(E)	90	150	240	
Flat	(F)	-	-	-	

3.1. Characterization of fabricated nanopatterns

The shape, dimensions and topographical variation of the fabricated nanopatterned substrates were characterized using AFM and SEM (Table 2-1, Figure 2-3) The AFM

analysis show uniform dimensional spacing (in nanometers) and shape divergence between the various TiO_2 coated gratings size as well as nanorectangles (Table 2-1).

For example, SEM analysis of the nanopatterns with dimension: 90 nm wide and 150 nm apart (Figure 2-3), each rectangle or gratings have the width of 90 nm and is separated from its neighbours by 150 nm (Figure 2-3). Thus, structural uniformity, shape and dimensional consistency were confirmed by these analyses.

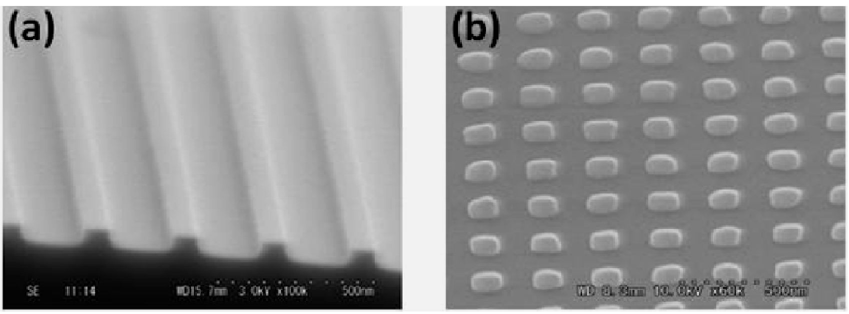


Figure 2-3: SEM of the nanopattern (total dimension 240nm) nanogratings (a) and nanorectangles (b) substrates.

3.2. Influence on functional protein expression

The natural ECM around hepatocytes inherently stimulates the expression of functional proteins such as albumin, transferrin, and cytochrome P-450. Therefore, to examine the effect of variations in nanopattern topography on the behaviors of HepG2 cells, and to identify which nano-features closely mimic the natural ECM and control the cell behavior, we observed the change in the expression level of these functional proteins using fluorescence immunostaining techniques.

3.2.1. Alteration in albumin expression

Fluorescence immunostaining of albumin was performed 12 hrs after cultuing the HepG2 on various nanopattern substrates, followed by the observation of green fluorescence using a fluorescence microscope (Figure 2-4). Shape modulation and dimensional alteration

of the nanostructures' topography by the expansion and reduction of the distance between different sizes of nanogratings significantly influenced the expression of albumin (green fluorescence). Gratings patterns with dimensions between 240 and 280 nm (Figure 2-4: A-B) and nanorectangles with 240nm total dimension (Figure 2-4E) exhibited higher albumin expression relative to HepG2 cells grown on flat surfaces (Figure 2-4F). In particular, HepG2 cells cultured on a 240 nm nanopattern (Figure 2-4A) showed a greater increase in albumin expression compared to cells cultured on other nanograting substrates with different dimensions. A significant decrease in the expression of albumin was observed upon alteration of geometry (rectangles instead of continuous lines) with the same dimensions (Figure 2-4: A, E).

A quantitative comparison of albumin expression is presented in Figure 2-5, in which the calculated fluorescence intensities obtained from HepG2 cells cultured on nanopattern substrates were statistically compared to the fluorescence observed from cells cultured on flat surfaces. Cells cultured on nanograting shapes showed a significant increase in albumin expression relative to the control ($p < 0.05$) as follow: total dimensions of between 240 nm (6 fold increase compared to control) and 280 nm (more than 4 fold increase compared to control) while nanorectangles showed 3 fold increase compared to control. Continuous nanogratings with a dimension of 240 nm significantly stimulated the expression of albumin compared to other nanogratings with various dimensional spacings, and the other shape with the same dimensional spacing ($p < 0.05$) (Figure 2-5). Thus, the results suggest that the 240 nm nanopattern with a continuous linear shape closely mimics the natural environment essential for the expression of albumin by HepG2 cells.

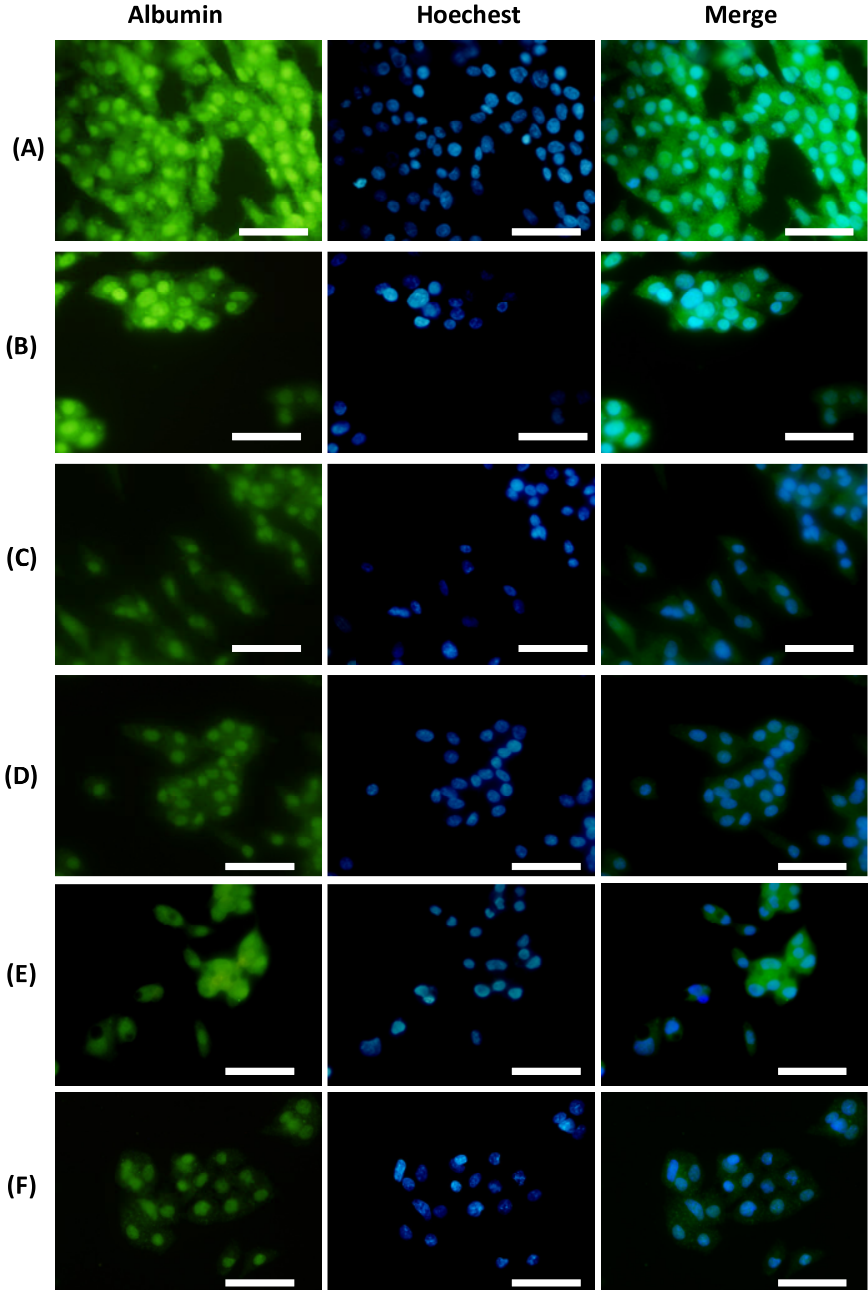


Figure 2-4: Immunofluorescent staining of albumin, a hepatic functional protein (green excitation ʎ = 488 nm)and nucleus using Hoechest (blue) in HepG2 cells cultured on different topographical nanopattern. The total dimensions of the grating shape: (A) = 240 nm, (B) = 280 nm, (C) = 310 nm, (D) = 410 nm, and (E) nanorectangles with a total dimension of 240 nm, finally (F) control or flat surface. (Scale bar=50um).

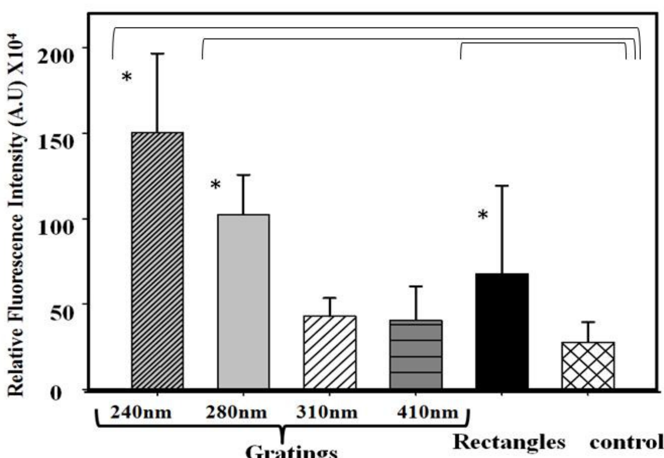


Figure 2-5: Relative mean fluorescence intensities calculated using image J for HepG2 cultured in different nanopattern surfaces and flat substrates.

3.2.2. Alteration in transferrin expression

The expression of the functional protein transferrin was determined from green fluorescence in the cells using an immunostaining technique. Figure 2-6 shows fluorescence images of the formed transferrin upon culturing of HepG2 on various nanopatterns and flat surfaces. 240 nm linear nanogratings substrate prompted the expression of transferrin compared to other flat surfaces and other nanopatterns(Figure 2-6A). The intermittent rectangle pattern resulted in only a slight increase in the expression of transferrin compared to the control (Figure 2-6E, F). Low transferrin expression was also observed in HepG2 cells cultured on nanogratings with different winder interspacing between these nanostructures (Figure 2-6: B-D). These results suggest that the level of transferrin is modulated by the simulation of natural ECM cues induced by 240 nm nanogratings only, and that other size of nanofeatures decrease the level of transferrin expression. A quantitative comparison of transferrin expression is presented in (Figure 2-6G), in which the calculated fluorescence intensities obtained from HepG2 cells cultured on such substrates were statistically compared to the fluorescence observed from cells cultured on flat surfaces. Cells cultured on

nanograting shape with total dimensions 240 nm showed a significant increase in transferrin expression relative to the control or flat surface ($p < 0.05$) as cells cultured on nanogratings 240 nm gratings shape has showed nearly 1.3 fold increase in transferrin expression compared to flat surfaces (Figure 2-6 G).

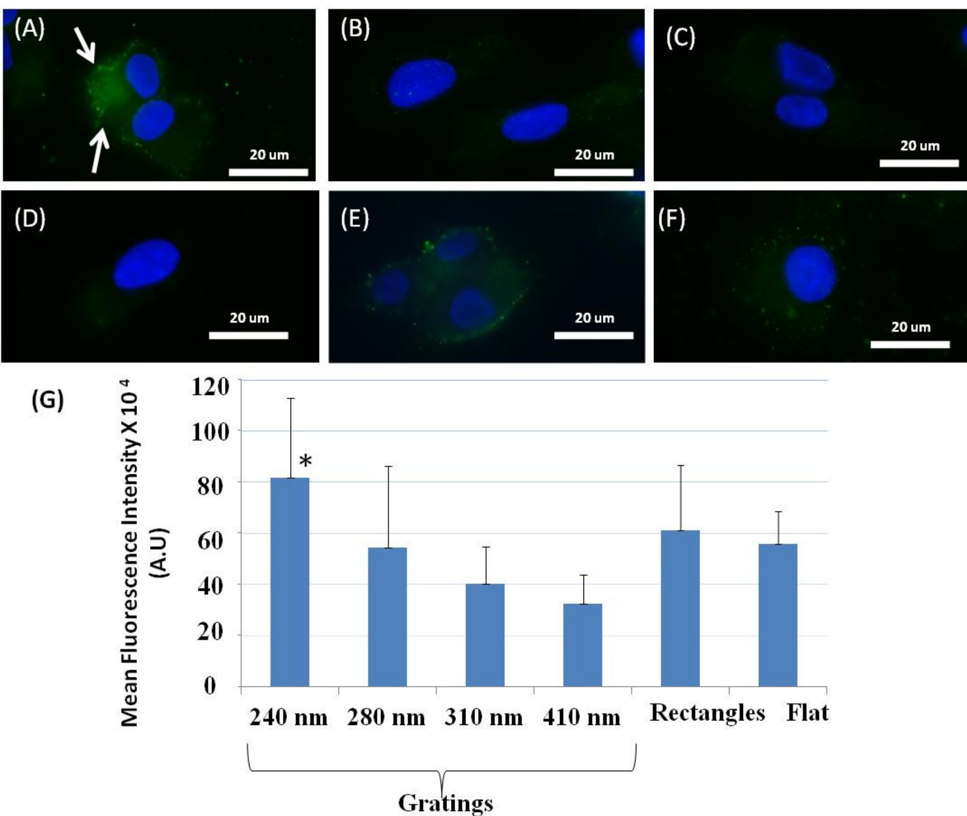


Figure 2-6: Immunofluorescent staining of the hepatic protein, transferrin (green excitation λ= 488 nm), and the nucleus (blue) in HepG2 cells cultured on different topographies. The nanogratings had dimensions of (A) = 240 nm, (B) = 280 nm, (C) = 310 nm, (D) = 410 nm, (E) nanorectangles with a dimension of 240 nm, (F) control or flat surface. (G) Mean fluorescence intensities calculated using image J for HepG2 cultured in different nanopattern surfaces.

3.2.3. Alteration in cytochrome P-450 expression

The expression of cytochrome P-450 was analysed after culturing of HepG2 cells on nanopattern substrates and flat surfaces. Figure 2-7 shows cytochrome P-450 as red fluorescence inside HepG2 cells cultured on our substrates after 18 hrs. The cells cultured on nanogratings with spacings of 240 nm and 310 (Figure 2-7: A, C) showed a significant increase in cytochrome P-450 compared to cells cultured on other topographical dimensional features and shapes, or on a flat surface (Figure 2-7B, D, E and F). These results suggest that cytochrome P-450 levels are stimulated by the topography of nanopattern with size 240 and 310 nm with continuously linear nanofeatures. A quantitative comparison of cytochrome P-450 expression is presented in (Figure 2-7G), on which topography with size 240 and 310 nm TiO_2 substrate showed significant increase (nearly 2 fold increase) in cytochrome P-450 expression relative to the control or flat surface ($p < 0.05$).

3.3. Influence on the structure of hepatocytes

Geometrical modulation of topography and changes in the shape of the nanopattern while maintaining the critical dimension (240 nm) influenced cellular orientation, cell alignment and elongation. These alterations in the cells, and its directional induced cellular orientations were examined by observing the rearrangement of actin filaments, morphology and integrin-mediated focal adhesion.

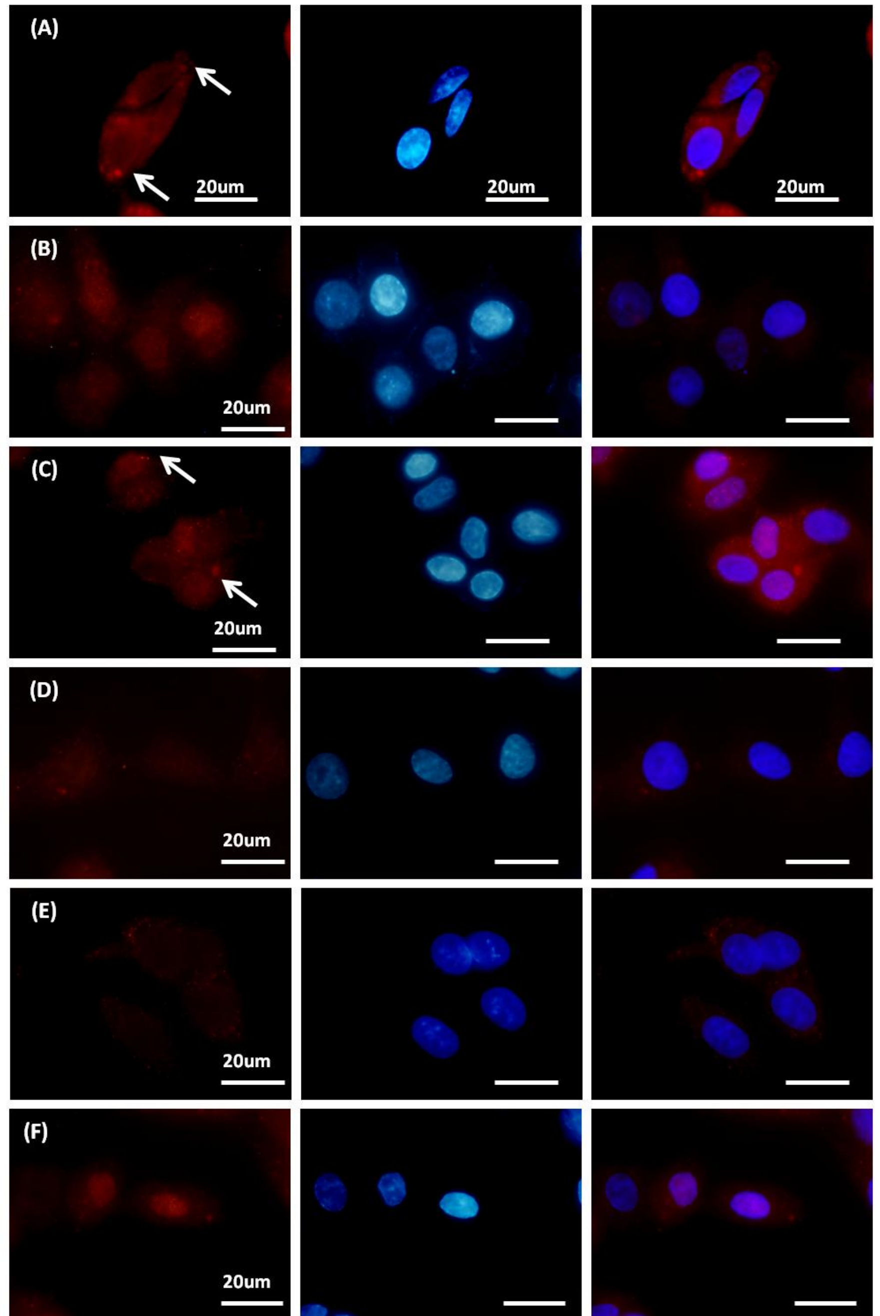


Figure 2-7: Immunofluorescent staining of Hepatic protein "Cytochrome P450 2C9" (Red excitation λ= 578 nm) in HepG2 cells cultured in different topographical shapes nanograting with total dimensions (A) = 240nm, (B) = 280 nm, (C)= 310 nm, (D)=410 nm, and (E) nanorectangels with 240 dimension compared to flat surfaces(F) Flat surface.

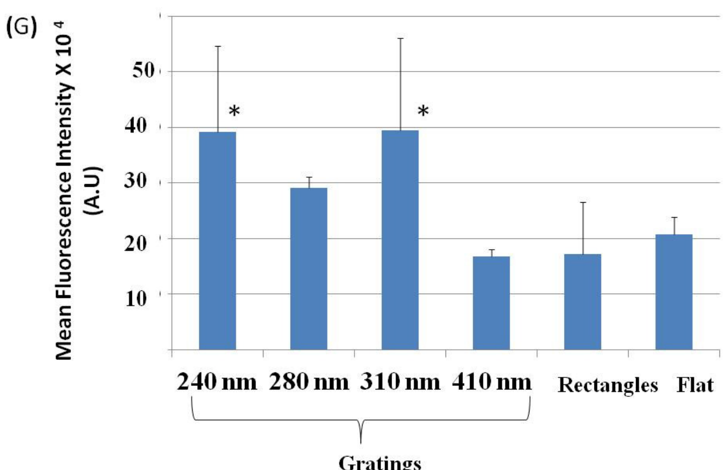


Figure 2-7 Continue: (G) Mean fluorescence intensities calculated using image J for HepG2 cultured in different nanopattern substrates and flat surface.

3.3.1. Alignments and actin filament rearrangements

To investigate the effects on the cytoskeleton by simultaneously altering the shape of the substrate nanofeatures while maintaining the same dimensions, the assembly and reorganizations of actin filaments were observed by culturing HepG2 cells on heterotropic and dimensionally well-defined nanograting/nanorectangle substrates. Figure 2-8, 2-9 shows the fluorescence staining of actin filaments, which indicates partial structural reorganization of hepatocyte cells to an orientation parallel to the nanogratings (Figure 2-8). In contrast, rectangular nanostructures with the same dimension (240 nm) enhanced the formation of filopodia and lamellapodia (Figure 2-9) but provided a less organized cytoskeleton. These alterations in the cellular framework 12 hrs after cell culturing were compared to cells grown on flat surfaces (Figure 2-9).

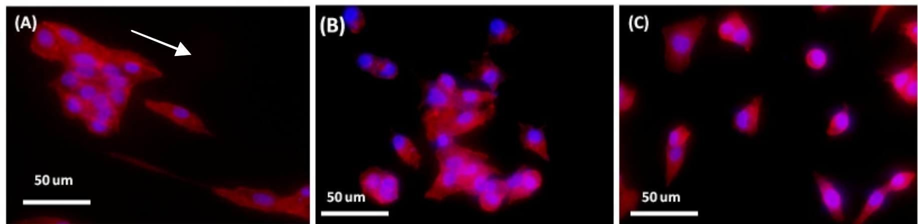


Figure 2-8: Cellular alignments of HepG2 cells visualized using actin staining after cultured for 12 hrs on in different substrates with topographical shape (A) nanogratings, (B)= nanorectangles, (C)=flat surfaces structures.

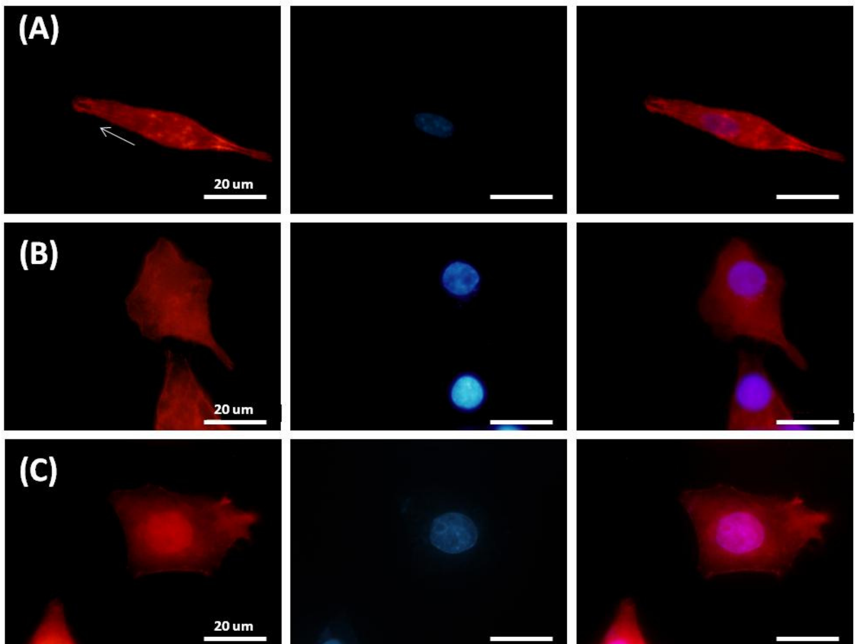


Figure 2-9: cytoskeletal rearrangement and actin filament rearrangement parallel to (A) gratings while (B)rectangles and (C) flat surface did not show such rearrangements.

3.3.2. Morphological variations examined using SEM

The morphological and alignment variations were confirmed with SEM. In 240 nm nanogratings, single cells aligned parallel to the longitudinal axes of nanogratings with the extension of filopedia over nanogratings or in the interspace between such gratings (Figure 2-10A). Single ells were likely stretched with line of axis in with filopedia perpendicular to nanogratings directions. Such cytoplasmic extension (filopodia) where anchored to both

nanopattern depressions and projections with lamellepodia reaching the lower surface of the interspace (Figure 2-10 A). While for rectangles cells did not show such parallel alignment with further extensions of filopedia in the interspace between such rectangles (Figure 2-10 B).

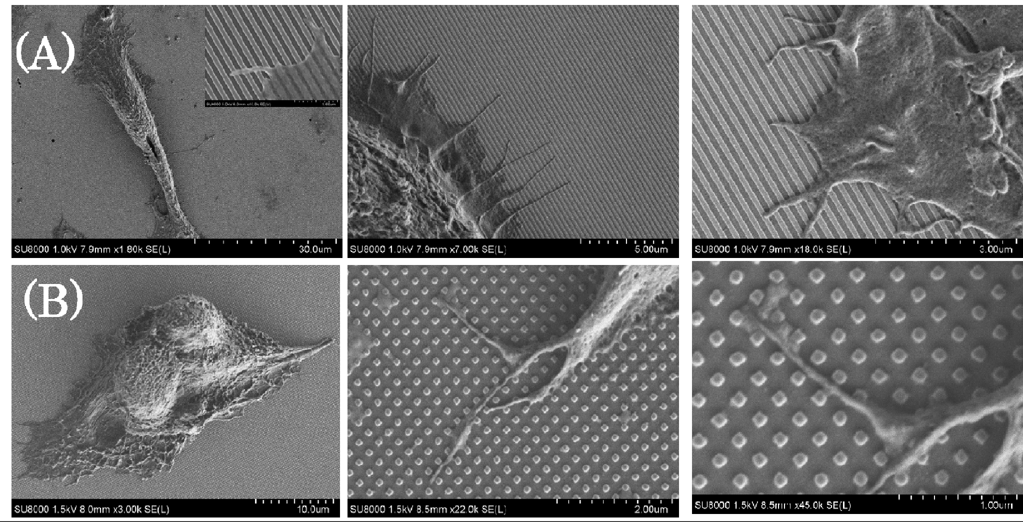


Figure 2-10: SEM images of HepG2 cells cultured for 12 hrs in (A) nanogratings compared to (B) nanorectangles which insets show the filopedia extention in the interspace between pattern and on top of it.

3.3.3. Integrin mediated focal adhesion

The formation of integrin β1 mediated focal adhesions was compared for cells grown on different nanopattern-shaped substrates with total dimension of 240 nm and compared to cells grown on a flat substrate. Comparisons were done using immunofluorescence staining of a transmembrane protein characteristic for focal contacts, integrin β1. Figure 2-11 shows the expression of integrin β1 as green fluorescence upon culturing of HepG2 cells over nanogratings and nanorectangles with 240 nm total dimensions and a flat surface substrate for 12 hrs. An increase in the integrin β1 formed was observed after culturing of HepG2 cells

on a continuous grating compared to cellson nanorectangles or on a flat substrate (Figure 2-11 A, C). Fewer integrin β1 clusters were expressed on the intermittent nanorectangles compared to the flat substrate (Figure 2-11 B, C). A quantitative comparison of integrin β1 expression is presented in (Figure 2-11 D) on which cells cultured on 240 nm continuous nanogratings showed a significant expression of integrin β1 (1.3 fold increase compared to nanorectangles and 2 fold increase compared to flat surfaces). Thus, 240 nm continuous nanogratings provided the nanocues for the recruitment and clustering of integrin β1 receptors.

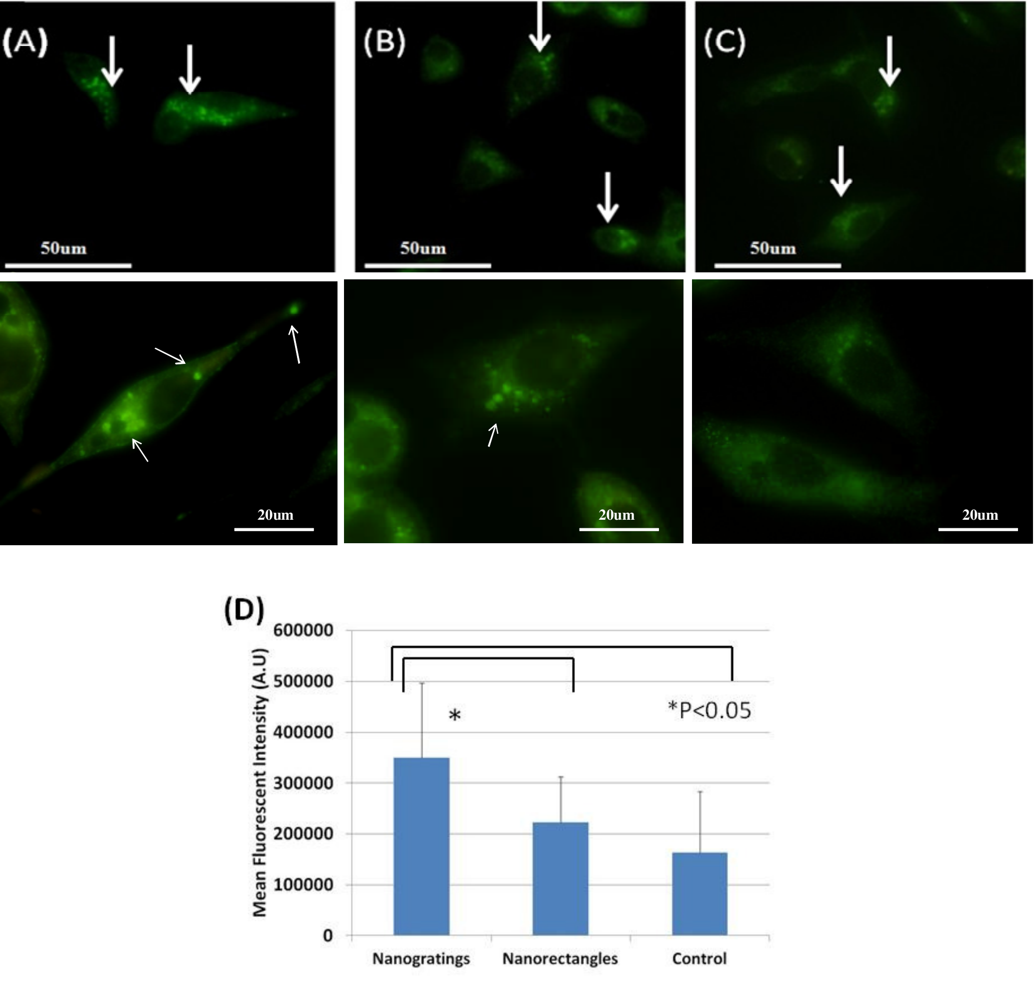


Figure 2-11: Study of integrin mediated focal adhesion formed using immunostaining

of Integrin β1 by Alexa-fluor 488 linked-AB, excitation ʎ488nm of HepG2 cells cultured on 240 nm (A) nanogratings, (B) nanorectangles (c)control (upper panel low magnification while lower panel higher magnification images). (D) Mean fluorescence intensities calculated using image J for HepG2 cells cultured in different nanopatterns.

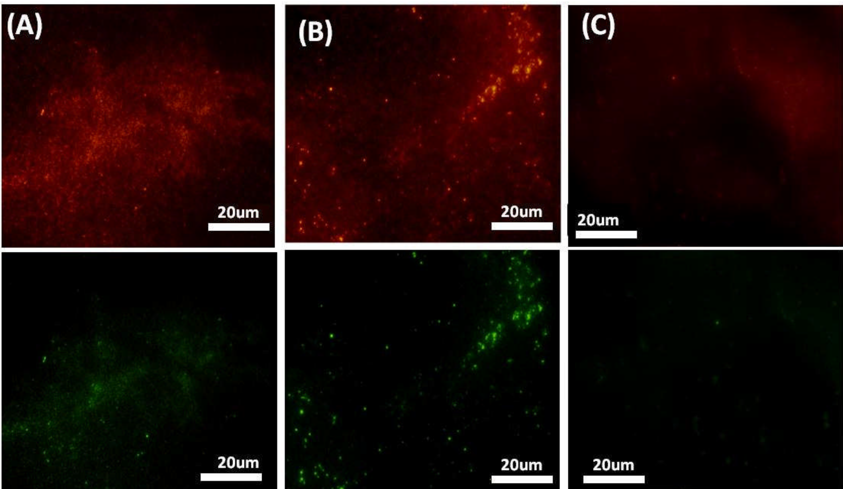


Figure 2-12: ECM components formed by HepG2 cells cultured for 66 hrs on nanopattern with different shapes and total topographical dimension 240nm (A) nanogratings (B) nanorectangles (c) control, on which red fluorescence represents the Collagen IV Ab (2ry- Alexa fluor-540), Green Fluorescence represent Fibronectin (2ry Ab Alexa-fluor-488) after decellularization using H_2O for 1 hr at 37 °C.

3.3.4. Native ECM Assembly

We propose that culturing HepG2 on nanopattern will have a considerable impact on the formation and assembly of natural ECM components such as fibronectin and collagen IV. Thus, a decellulariztion procedure was performed using H_2O, as previously reported, then the change in the formation and degree of assembly of fibronectin and collagen IV were examined using an immunostaining technique. Figure 2-12 shows the increase in the level of naturally assembled fibronectin and collagen IV after culturing and decelluarization of HepG2 cells on 240 nm nanopatterns with different shapes relative to the flat surfaces. The construction and assembly of two native ECM components, fibronectin and collagen IV,

were considerably stimulated 66 hrs after culturing HepG2 cells on nanopattern containing linear nanofeatures compared to cells seeded on rectangular nanostructures or on a flat surface (Figure 2-12). These results suggest that nanopattern with linear shape nanofeatures (total dimension of 240 nm) provided essential cues to increase the magnitude and assembly of naturally-produced ECM components.

Experimental & methodology

4.1. Fabrication of nanopattern substrates using electron beam lithography and atomic layer deposition

Cleaned Si (100) substrates were coated with ZEP520A resist (Nippon Zeon Co., Japan) and a thinner (anisol) at a ratio of 1:2 using a spin coater (Mikasa 1H-D7) at 6000 rpm. Prebaking was done at 180 °C for 3 min, followed by spin coating with a very thin layer of conductive material (10-20 nm) (Espacer; Syowa Denko Co., Japan) at 2000 rpm. The substrate was then irradiated with an e-beam (Elionix ELS-7500EX) with acceleration voltage of 50 kv and an I beam amperage of 220 pA. The substrate was then developed in 3 steps using H_2O, n-amyl acetate and mixture of methyl isobutyl ketone (89%)/isopropyl alcohol (11%) (Wako Co., Japan) and dried with N_2 gas. The precise size of fabricated substrate resulted from each e-beam was confirmed using SEM. An etching step was performed using inductive coupled plasma-reactive ion etching at 50W (sulphur hexafluoride 2.5 sccm + methyl tetrafluoride 3.5 sccm) with a total pressure of 0.1 Pa for 101 s. Next, the resist was removed using O_2 plasma, DMAC (dimethyl acetamide) and SPM (H_2SO_4 + H_2O_2, 3:1) respectively. The next steps included coating with a photoresist (AZ-5214E), UV irradiation with a photomask, reversal baking at 120 °C, and flood exposure to UV. The substrate was then developed using NMD-3 (2.38% TMAH) (tetramethyl ammonium

hydroxide) (Wako Co. Japan) for 1 min, then rinsed with H_2O. Finally, atomic layer deposition was conducted (Picosun SUNALE R-150). The deposition pressure inside the chamber was (500 Pa) at a temperature of 100 °C. The thickness of the TiO_2 layer was controlled by the number of cycles: 70 cycles gave a thickness of 5 nm. The TiO_2 precursor [tetra(dimethlyamino)titanate] was pumped into the chamber, followed by argon gas to remove undeposited precursor. Next, H_2O vapour was pumped in to form the inorganic TiO_2 layer from the organic precursor, then argon gas was pumped in to remove residual H_2O (Figure 2-13). Fabricated nanopatterns coated with thin films of TiO_2 were characterized using a scanning electron microscope (Hitachi-S3000N) and an atomic force microscope (SIIL-trace). Further experiments were done using the fabricated substrates after dry heat sterilization of the substrate at 170 °C for 1 hr.

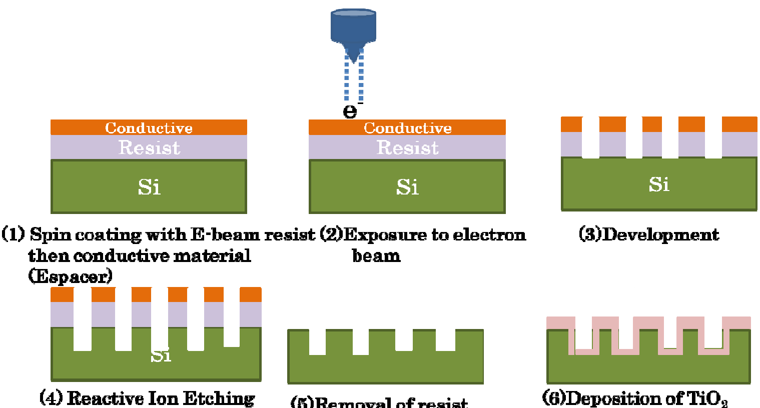


Figure 2-13: Scheme illustrates the steps required for the fabrication nanopatterns with deposited TiO_2.

4.2. Hepatic cell culture

HepG2 cells were cultured in Dulbecco's MEM (Nacalai Tesque, Kyoto, Japan) with 10% heat inactivated FBS and supplemented with 100U penicillin/ 100 µg streptomycin (Nacalai Tesque, Kyoto, Japan) per ml medium. All cells were maintained at 37 °C in a 100% humidified atmosphere under 5% CO_2. At 70-80% confluence, cells were trypsinized

and seeded over the nanopattern surfaces for 12 to 18 hrs. For the expression of ECM components, cells were cultured for 66 hrs; a fresh medium was added after 33 hrs.

4.3. Immunostaining and fluorescence detection

HepG2 cells cultured on nanopatterned substrates were fixed for 15 min in 4% p-formaldehyde (PFA) in PBS at 4 °C. Excess aldehyde was quenched using 0.1 M glycine for 5 min. Then, the cells were permeabilized in 1% triton X-100 in PBS for 5 min, then primary antibodies were added. The antibodies used : rabbit polyclonal anti-albumin antibody- C -terminal (diluted 1:25; Abcam, Cambridge, UK), rabbit polyclonal anti-transferrin antibody (diluted 1:500; Abcam), mouse monoclonal anti-cytochrome P-450 2C6 antibody (1 μg/ml; Abcam), CD29 mouse anti-human mAb-ALexa Fluro® 488 conjugate for intigrin β1 (diluted 1:100; Invitrogen Life Technologies, Eugene Oregon, USA). These antibodies were incubated with the cells at 4 °C overnight. Even though we used a medium with 10% fetal bovine serum which may contain proteins as albumin, there is no interference or cross reactions with the primary antibodies we used, as these antibodies are specific for human proteins not bovine species. Then, secondary antibodies were added for 1 hr. These secondary antibodies were Alexa Fluor ® 568 goat anti-mouse IgG (H + L) (diluted 1:500; Invitrogen Life Technologies Eugene Oregon, USA) and goat polyclonal anti-rabbit IgG - H&L-DyLight® 488 (diluted 1:500; Abcam). The cells were observed using an upright fluorescence microscope (Olympus BX51) equipped with an Olympus DP70 digital camera. DP Controller Ver. 3.1.1 was used to process the images. For quantitative comparison of albumin expression, we used the freeware image analysis software ImageJ, as previously reported [24]. The cell area was determined by manual delineation of raw fluorescence images. A minimum of 12 cells were analysed from two independent experiments. Cytoskeletal F-actin was visualized by treating the cells with

phalloidin – TRIC (Sigma-Aldrich, Germany) at 5 μg/ml for 15 min. Nuclei were stained using Hoechest 33342 at 5 μg/ml for 10 min.

To investigate ECM components, decelluarization was performed as previously reported [25]. Cells were cultured on nanopattern surfaces with various shapes for 66 hrs, then incubated with 2 ml of distilled water for 1 hr at 37 °C for cell lysis. A washing step by careful immersion in PBS was done 3 times. The deposited ECM was fixed using 4% PFA for 20 min. Primary antibodies such as mouse monoclonal antifibronectin (diluted 1:50; Santa Cruz Biotechnology) and mouse monoclonal anticollagen IV (diluted 1:50; Santa Cruz Biotechnology) were added for 30 min, then secondary antibodies were added for 30 min. The secondary antibodies were goat anti-mouse IgG-TR (diluted 1:250; Santa Cruz Biotechnology) and Alexa Fluor® 488 goat anti-mouse IgM (H+L) (diluted 1:250, (Invitrogen Life Technologies)). The effective removal of cells and cellular debris by this method was verified by fluorescent labelling of the decellularized nanopattern surfaces with Hoechest 33342.

4.4. Scanning electron microscope investigation

Investigations of the cell rearrangements and alignments were done by scanning electron microscopy (SEM) using (Hitachi S-4800, Japan). Prior to SEM investigations the samples were prepared as follows. At the end of the culture period the cells on the nanopattern were fixed with 2.5 vol% glutaraldehyde (Wako, Japan) in PBS for 2 h. After which the cells were dehydrated through a series of ethanol concentrations (10%, 40%, 60%, 80%, 100%) for 5 min, respectively. Final desiccation was done using freeze drying (Hitachi Es 2030, Tokyo, Japan). Finally, the samples were placed on SEM specimen holders and observed under field-emission scanning electron microscope operated at an acceleration voltage in the range 1–5 kV.

5. Discussion

One challenge in tissue engineering is the construction of a biomaterial that mimics the native *in-vivo* micro/nanoenvironment while preserving cellular viability and functionality. Many reports have focused on the discrepancies in cellular behaviors, which are highly dependent on the type of human cell and the surface characteristics of the biomaterial [20-23].

To address these challenges, we constructed various surfaces with different shapes and heterotropic dimensions. The multiple patterns mimic the nanocues provided by naturally occurring ECM components to control and regulate numerous hepatocyte functions. Thus, grating shapes and intermittent nanorectangles that could mimic hierarchically-extended collagen nanofibrillar structures were generated using electron beam lithography and atomic layer deposition. The distance between nanogratings was 150, 220 or 320 nm, and the width of the nanogratings was either 90 or 120 nm. The dimensions of the nanofeatures was chosen to fit the size range of assembled collagen fibrils 260-410 nm [26] found in cells in their native environment. While the height was chosen 40 nm as an increase in the z scale dimension of protruding nanofeatures has been shown to impair cellular behaviors in different cells [20-23, 27]. Furthermore, such a larger height would result to restrict the attachment of cells to upper surface of nanogratings..

Our results suggest several outcomes. First, the change in the planer area between the nanogratings of bio-inspired surfaces resulted in changes in hepatocellular functionality. Second, we demonstrate that the loss of collagen-like fibrillar structures by changing from a continuous linear shape to an intermittent shape or to rectangles considerably altered the

cellular functionality of HepG2. Third, our findings suggest that the control of the physical nanocues associated with a certain dimension and shape that closely mimics the natural ECM could be critical for control the cellular behavior.

We focused on determining the functional responses of HepG2 cells to several topographic features with various dimensions and shapes. The expression levels of albumin, transferrin and cytochrome P-450 were tested as functional makers. Albumin, a liver-specific marker, was investigated in HepG2 cultured on different nanopattern. Specific nanopattern that likely mimicked the matrix configuration (total lateral dimension of 240 nm) showed higher albumin expression. Likewise, nanopattern with this specific lateral dimension supported a higher level of two other functional proteins, cytochrome P-450 and transferrin.

Most of previous research have focused on the study of total features dimensions (depth or width) $\geq$ 350 nm and up to microns [28-30], while a few research only tried to study the influence of smaller dimensional features or the role of diminished scale lateral features on different cell behaviors. Nanogratings total dimension equivalent to 210 nm was able to induce effective alteration in the alignment and orientation of glioma cells [31]. Furthermore, osteoblast morphologically recognize surface topography starting from 75 nm with resultant alteration in the alignments and deposition of mineralized ECM components, while 150 nm features size confirmed to start the induction of osteoblast-specific genes expression [31] (Alkaline phosphatase, osteocalcin, and bone sialoprotein). Moreover, the expression of IL-1β and TNF-α from murine macrophage *in-vivo* and *in-vitro*, was upregulated on nanogratings starting from 150 nm lateral dimension**,** on which these signalling proteins play considerable role in the wound healing process [33]. Such topography, features size <350 nm, in particular likely to highly mimics the variable distance between individual collagen fibrils since collagen fibrils size are varying in diameter

260-410 nm as previously reported [26]. That allows the cell/surface communication with subsequent increase in cellular functionalities.

Previously, anisotropically dimensional nanostructures with gratings /interspace 1:1 were used, the increase in the lateral dimensions even up to up to micro–scale resulted in an increase in the cellular functions [20-23]. However, our results suggested that an increase in the nanofeatures, gratings/interspace from ≃ 1:2 (90:150 nm) into ≃ 1:3 (90:220 nm) or ≃ 1:4 (90:320 nm) has been associated with a decrease in expression of some tested functional proteins (i.e. albumin). A recent research reported that the increase in the interspace between nanogratings into 1:5 (300:1500 nm) would significantly decrease the differentiation of human neuronal stem cells with the decrease in expression of specific neuronal biomarkers [30]. These alterations in the behaviors of cells over topographical features could be reasoned by 2 facts: the cell type is a key element in cell-material interactions, as different cells behave diversely when cultured in the nano/submicro-scale nanofeatures [34]. Secondly, the cells are sensitive to topographical alterations, as the same cells could behave differently by the modification in the feature specific dimensions despite fixing the lateral dimension and shape. For instance, a change in the alignment percentage of corneal epithelial cells is observed when altering the gratings/interspace dimension from 1:4 (70:330) into 1:2 (180:220) for nanogratings with 400nm lateral dimension [35, 36]. Consequently, the nanostrcutures lateral dimension (240 nm) likely supports the cellular orientation and integrity of hepatocytes and allows the control of their behaviors.

The other geometry investigated, nanorectangles, resulted in a decrease in the formation of albumin, transferrin and cytochrome P-450 compared to cells attached to the continuous striated form of the same size. Thus, the higher the degree of resemblance between the superficial topographical cues on nanopattern and natural ECM, the better the

cytocompatibility and functionality of HepG2 grown on such nanostructures.

Our results suggest that the topographical characteristics of nanocues of well-defined dimension and shape (240 nm with a striated appearance) can control and regulate multiple hepatocellular functions. While other dimensions resulted in increased expression of hepatic proteins, such as the 310 nm dimension for gratings for cytochrome P-450 expression (all as compared to the control), the simultaneous maintenance of elevated levels of several hepatic functions relies on sophisticated signals. These entangled signals are organized to some extent by diversified physical and biochemical cues. The underlying mechanisms for this enhanced functionality have yet to be fully revealed, and needed to be explored in the future. These studies will provide an in-depth understanding of the relationship between ECM-like topography and liver cell functionality, and thus will potentially be of great benefit for tissue engineering and its future applications.

HepG2 cultured in nanograting substrates displayed a fundamentally different morphology compared to cells cultured on nanorectangles or flat surfaces. On continuous nanogratings, around 60-70% of the single cells are aligned in the longitudinal direction, parallel to the nanogratings, and exhibit aligned cellular morphology. Cells cultured on intermittent rectangles or flat surfaces did not show this parallel alignment. The forementioned extension of actin filaments result from the fixation of filopodia as anchoring points in the planer area between the gratings and over it on which these anchoring points can initiate the recruitment of integrins and other adhesion proteins. So it is likely will facilitate the orientation and formation of focal adhesion formed [37]. These cytoplasmic extensions not only may be involved in the explorations of the material surfaces but it may also play a role in the alteration of spatial conformation of adhesion complex by acting as a bridge between external surfaces and cells. Although we have no direct evidence that

correlates the observed structural changes with production of functional proteins, It is expected that these changes in the cellular morphology and cytoarchitecture would be transduced via the provoke of wide arrays of chemical signalling pathways with further change in gene expression and cellular functionalities. In contrast, nanorectangles with no alignment while guiding the formation of more filopodia and lamellapodia compared to the control. This suggests that the filopodia can explore the surroundings of the nanorectangles where focal adhesion assembly can be occurred

While a diverse variety of cellular behaviors are influenced by integrins mediated signals, the recruitment and clustering of integrins usually occurs by a vast number of extracellular and intracellular stimuli for the anchoring and focal adhesion formation [38, 39]. Integrins are heterodimeric receptors that are responsible for transmembrane signal transduction from the surrounding native ECM to the cell. The extracellular domain of integrin binds to specific motifs in the ECM, while the cytoplasmic domain is associated with the actin cytoskeleton and other affiliated proteins. For example, integrin β1 receptors are likely involved in the maintenance and development of specific cell architectures. The multiplicity and functionality of integrin β1 expression in hepatic cells such as HepG2 has been previously reported [40]. Nnaopattern with a striated appearance altered the expression of integrin β1 more than the other substrates (nanorectangles and a flat surface), suggesting that the modification in physical traits of substrate could be responsible for alteration in cellular behaviors. This is in correspondence with the previously reported research, in which Integrin α2β1 receptors recognize collagen fibrils with a highly ordered architecture more than collagen monomers with only unique peptide motif consequently altered the cellular adhesion [41].

The influence of the nanopattern on naturally formed and assembled ECM was

investigated, since HepG2 cells innately deposit and assemble fibrillar dense network of collagen IV and fibronectin [42]. The investigation collagen IV and fibronectin natively assembled was performed (Figure 2-12). Both proteins were preferentially deposited and assembled in between the nanogratings compared with nanorectangles with a total dimension of 240 nm or with flat surfaces. This suggests that the striated appearance of the surface, which resembles native collagen fibrils in shape and dimensions, was responsible for the abundant assembly of ECM components, and that HepG2 cells recognized this bio-inspired mimic of native ECM and reacted accordingly. Consequently, the superficial characteristics of a biomaterial are an important tool for manipulating and controlling the formation of highly organized ECM.

6. Conclusions

In summary, we fabricated diversified nanopatterns with specific superficial topographical nanofeatures of various sizes and shapes using electron beam lithography and atomic layer deposition. Our results suggest that the interactions of a mammalian hepatic cell line with nanogratings, which have heterotropic dimensions, may regulate cellular function. Additionally, we confirmed that alteration of the shape of nanofeatures while maintaining the same dimensional characteristics could be an effective tool to control the cellular behavior. For example, alteration of collagen fibrillar-like structures from a continuous linear shape to intermittent rectangles lowered the cellular functionality of HepG2 cells. Finally, our findings suggest that physical nanocues, such as edge to edge spacing and feature models, must be controlled for a higher hepatic functionalities in-vitro. These finding could be utilized in the design of facile and reproducible surfaces with distinct functionalities. Identification of the best topographical dimensions and configurations for hepatocyte-based

biomaterials might provide the basis for the fabrication of specialized surfaces for implantable bioreactors to treat liver failure, or the expression of therapeutic proteins.

7. References

[1] Curtis ASG, Gadegaard N, Dalby MJ, Riehle MO, Wilkinson CDW, Aitchison G, Cells react to nanoscale order and symmetry in their surrounding, IEEE Trans Nanobiosci., 2004; 3: 61-65.

[2] Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CDW, Oreffo ROC, The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder, Nature Mater., 2007; 6: 997-1003.

[3] You M-H, Kwak MK, Kim D-H, Kim K, Levchenko A, Kim D-Y, Suh K-Y, Synergistically Enhanced Osteogenic Differentiation of Human Mesenchymal Stem Cells by Culture on Nanostructured Surfaces with Induction Media, Biomacromolecules, 2010; 11: 1856-1862.

[4] Lukashev ME, Werb Z, ECM signalling: orchestrating cell behavior and misbehavior, Trends Cell Bio., 1998; 8: 437-441.

[5] Pamula E, De Cupere V, Dufrene YF, Rouxhet PG, Nanoscale organization of adsorbed collagen: Influence of substrate hydrophobicity and adsorption time, J. Colloid Interface Sci., 2004, 271, 80-91.

[6] Bozec L, van derHeijden G, Horton M, Collagen Fibrils: Nanoscale Ropes, Biophys. J., 2007; 92:70-75.

[7] Wang N, Ingber DE, Control of cytoskeletal mechanics by extracellular matrix, cell shape, and mechanical tension, Biophysical J., 1994; 66: 2181-2189.

[8] Abrams GA, Goodman SL, Nealy PF, Franco M, Murphy CJ, Nanoscale topography of the basement membrane underlying the corneal epithelium of the rhesus macaque, Cell Tissue Res., 2000, 299, 39-46.

[9] Hornberger LK, Singhroy S, Cavalle-Garrido T, Tsang W, Keeley F, Rabinovitch M, Synthesis of Extracellular Matrix and Adhesion Through β1 Integrins Are Critical for Fetal Ventricular Myocyte Proliferation, Circulation Res., 2000; 87: 508-515.

[10] Wu C, Dedhar S, Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes, J. Cell Bio., 2001; 155: 505-510.

[11] Biggs MJP, Ritchard RG, Dalby MJ, Nanotopographical modification: a regulator of cellular function through focal adhesions, Nanomed: Nanotechnol Biol Med., 2010; 6: 619-633

[12] Mandes PM, cellular nanotechnology mimicking biological interfaces and smarter, Chem Soc Rev., 2013; 42: 9207-9218.

[13] Takayama G, Taniguchi A, Okano T, Identification of differentially expressed genes in hepatocyte/endothelial cell co-culture System, Tissue Eng., 2007, 13, 159-166.

[14] Bokhari M, Carnachan RJ, Cameron NR, Przyborski SA, Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function during toxicological challenge, J. Anat., 2007; 211: 567-576.

[15] Nishida Y, Taniguchi A, Induction of albumin expression in HepG2 cells using immobilized simplified recombinant fibronectin protein, In-vitro Cellular & Developmental Biology - Animal, 2013; 49: 400-407.

[16] Hoessa A, Teuschera A, Thormanna A, Aurichb H, Heilmanna A, Cultivation of hepatoma cell line HepG2 on nanoporous aluminum oxide membranes, Acta Biomaterialia, 2007; 3: 43-50.

[17] Nakazawa K, Lee S-W, Fukuda J, Yang D-H, Kunitake T, Hepatocyte spheroid formation on a titanium dioxide gel surface and hepatocyte long-term culture, J Mater Sci: Mater Med., 2006; 17: 359-364.

[18] Anselme K, Osteoblast adhesion on biomaterials, Biomaterials, 2000; 21: 667-681.

[19] Kubo K, Tsukimura N, Iwasa F, Ueno T, Saruwatari L, Aita H, Chiou W, Ogawa T, Cellular behavior on TiO_2 nanonodular structures in a micro-to-nanoscale hierarchy model, Biomaterials, 2009; 30: 5319-5329.

[20] Wang P-Y, Yu J, Lin J-H, Tsai W-B, Modulation of alignment, elongation and contraction of cardiomyocytes through a combination of nanotopography and rigidity of substrates, Acta Biomater., 2011; 7: 3285-3293.

[21] Myrna KE, Mendonsa R, Russell P, Pot SA, Liliensiek SJ, Jester JV, Nealey PF, Brown D, Murphy CJ, Substratum Topography Modulates Corneal Fibroblast to Myofibroblast Transformation, IOVS., 2012; 53: 811-816.

[22] Tsai W-B, Lin J-H, Modulation of morphology and functions of human hepatoblastoma cells by nano-grooved substrata, Acta Biomater., 2009; 5: 1442-1454.

[23] Prodanov L, Lamers E, Domanski M, Luttge R, Jansen JA, Walboomers XF, The effect of nanometric surface texture on bone contact to titanium implants in rabbit tibia, Biomaterials, 2013; 34: 2920-2927

[24] Burgess A, Vigneron S, Brioudes E, Labbé J-C, Lorca T, Castro A, Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance, Proc Natl Acad Sci., 2010; 107: 12564-12569.

[25] Herrema H, Czajkowska D, Théard D, van der Wouden JM, Kalicharan D, Zolghadr B, Hoekstra D, van IJzendoorn SCD, Rho Kinase, Myosin-II, and p42/44 MAPK Control Extracellular Matrix-mediated Apical Bile Canalicular Lumen Morphogenesis in HepG2 Cells, Mol Bio Cell., 2006; 17: 3291-3303.

[26] Bettinger CJ, Kulig KM, Vacanti JP, Nanofabricated Collagen-Inspired Synthetic Elastomers for Primary Rat Hepatocyte Culture, Tissue Eng: Part A, 2009;15: 1321-1329. Robert Langer, Sc.D.,4 and Jeffrey T. BorensteinZhu B, Lu Q, Yin J, Hu J, Wang Z, Alignment of osteoblast-like cells and cell-produced collagen matrix induced by nanogrooves, Tissue Eng., 2005; 11: 825-834; Perumal S, Antipova O, Orgel JP, Collagen fibril architecture, domain organization, and triple-helical conformation govern its proteolysis, Proc Natl Acad Sci., 2008; 105: 2824-2829.

[27] Biela SA, Su Y, Spatz JP, Kemkemer R, Different sensitivity of human endothelial cells, smooth muscle cells and fibroblasts to topography in the nano–micro range, Acta Biomaterialia, 2009; 5: 2460-2466.

[28] Pan F, Zhang M, Wu G, Lai Y, Greber B, Schöler HR, Chi L, Topographic effect on human induced pluripotent stem cells differentiation towards neuronal lineage, Biomaterials, 2013; 34: 8131-8139.

[29] Kulangara K, Yang Y, Yang Y, Leong KW, Nanotopography as modulator of human mesenchymal stem cell function, Biomaterials, 2012; 33: 4998-5003.

[30] Yang K, Jung K, Ko E, Kim J, Park KI, Kim J, Cho S-W, Nanotopographical Manipulation of Focal Adhesion Formation for Enhanced Differentiation of Human Neural Stem Cells, ACS Appl Mater Interfaces., 2013; 5: 10529-10540.

[31] Zhu B, Zhang Q, Lu Q, Xu Y, Yin J, Hu J, Wang Z, Nanotopographical guidance of C6 glioma cell alignment and oriented growth, Biomaterials, 2004; 25: 4215-4223.

[32] Lamers E, Walboomers XF, Domanski M, te Riet J, van Delft FCMJM, Luttge R, Winnubst LAJA, Gardeniers HJGE, Jansen JA, The influence of nanoscale grooved substrates on osteoblast behavior and extracellular matrix deposition, Biomaterials, 2010; 31: 3307-3316.

[33] Lamers E, Walboomers XF, Domanski M, Prodanov L, Melis J, Luttge R, Winnubst L, Anderson JM, Gardeniers HJGE, Jansen JA, In-vitro and *in-vivo* evaluation of the inflammatory response to nanoscale grooved substrates, Nanomed: Nanotechnol Bio Med., 2012; 8:308-317.

[34] Biela SA, Su Y, Spatz JP, Kemkemer R, Different sensitivity of human endothelial cells, smooth muscle cells and fibroblasts to topography in the nano–micro range, Acta Biomater., 2009; 5: 2460-2466.

[35] Fraser SA, Ting Y, Mallon KS, Wendt AE, Murphy CJ, Nealey PF, Sub-micron and nanoscale feature depth modulates alignment of stromal fibroblasts and corneal epithelial cells in serum-rich and serum-free media, J Biomed Mater Res Part A., 2007; 86A: 725-735.

[36] Teixeira AI, Abrams GA, Bertics PJ, Murphy CJ, Nealey PF, Epithelial contact guidance on well-defined micro- and nanostructured substrates, J. Cell Sci., 2003; 116: 1881-1892.

[37] Loesberg WA, te Riet J, van Delft FCMJM, Schön P, Figdor CG, Speller S, van Loon GGWA, Walboomers XF, Jansen JA, The threshold at which substrate nanogroove dimensions may influence fibroblast alignment and adhesion, Biomaterials, 2007; 28: 3944-3951.

[38] Luque A, Gomez M, Puzon W, Takada Y, Sanchez-Madrid F, Cabanas C, Activated Conformations of Very Late Activation Integrins Detected by a Group of Antibodies

(HUTS) Specific for a Novel Regulatory Region(355-425) of the Common β1 Chain, J Biol Chem., 1996; 271:11067-11075.

[39] Gomez M, Luque A, del Pozo MA, Hogg N, Sanchez-Madrid F, Cabanas C, Functional relevance during lymphocyte migration and cellular localization of activated β1 integrins, Eur J Immunol., 1997; 27: 8-16.

[40] Matsumotu A, Arao S, Otsuki M, Role of β1 integrins in adhesion and invasion of hepatocellular carcinoma cells, Hepatology,1999; 29: 68-69.

[41] Jokinen J, Dadu E, Nykvist P, kapyla J, White DJ, Ivaska J, Vehvilainen P, Reunanent H, Larjava H, Hakkinen L, Heino J, Integrin-mediated Cell Adhesion to Type I Collagen Fibrils, J Biol Chem., 2004; 279: 31956- 31963.

[42] Bouma M-E, Rogier E, Verthier N, Labarre C, Feldmann G, Further cellular investigation of the human hepatoblastoma-derived cell line HepG2: Morphology and immunocytochemical studies of hepatic-secreted proteins, In-vitro Cell Develop Bio., 1989; 25: 267-275.

Chapter 3

Integrations of nanopattern (Physical) with RGD (Chemical) variations that mimic ECM cue to control hepatocellular behaviors

1. Summary

Physical topographical features and/or chemical stimuli to the extracellular matrix (ECM) provide essential cues that manipulate cell functions. From the physical point of view, contoured nanostructures are important for the cell behavior in general, and for cellular functions. From the chemical point of view, ECM proteins containing an RGD sequence are known to alter cell adhesion and functions. In this study, the influence of integrated physical and chemical cues on a liver cell line (HepG2) was investigated. To mimic the physical cues provided by the ECM, nanogratings with specific dimensional and geometrical characteristics (nanogratings 90 nm wide and 150 nm apart) were fabricated. To mimic the chemical cues provided by the ECM, The immobilization of the RGD motif on the surface of such nanostructures. The hepatic cell line morphological and functional changes induced by simultaneously combining these diversified cues were investigated, including cellular alignment and the expression of different functional proteins. The combination of nanopatterns and surface modification with RGD induced cellular alignment and expression of functional proteins, indicating that physical and chemical cues are important factors for increasing hepatocyte function.

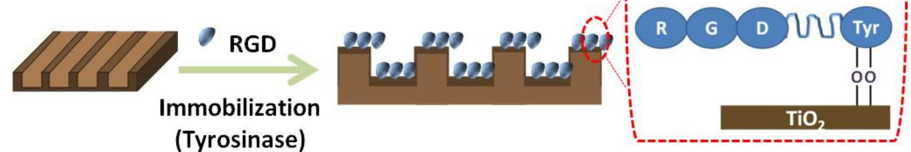


Figure 3-1: Manipulation of physical and chemical characteristics of susbtrates by the integration of topography with the immobilization adhesive RGD on TiO2 thin films using Enzyme catalyzed reaction.

2. Background

Living cells in their native environment are embedded in a complex of well-defined organic material and macromolecules called the extracellular matrix (ECM). The ECM assembles into a three-dimensional mesh with precisely contoured nanostructures [1]. The architecture and composition of the ECM provide the essential physical cues and chemical factors that trigger and control cellular behaviors [2]. This is particularly true for liver cells, as they need a specific architecture (avoid flat architecture with 3D cubic configuration) with distinctive surroundings for the maintenance of innate cellular functionalities as the production of hepatic proteins, metabolic and detoxifying functions. A decrease in hepatocellular functionalities generally occurs when liver cell lines are cultured on two-dimensional surfaces due to the absence of ECM proteins and cues. Collagen is an important protein of ECM that forms a triple helix structure to generate fibers approximately 300 nm long and 1.5 nm wide [3,4]. In addition to surface topography, the chemical composition of the ECM plays an essential role in the control of cellular behavior [5–7] through integrin-mediated signaling pathways [8,9]. Integrins are heterodimeric transmembrane surface receptors that mediate connections between cells and the ECM. Several ECM proteins activate integrins through different mechanisms, resulting in the integration of multiple signaling pathways and thus different cellular behaviors [10,11]. Fibronectin, for example, is an ECM protein with a specific peptide sequence, Arginine-Glycine-Aspartic acid (RGD), that alters cellular attachment and spreading, as well as cell integration properties [12] The associations of peptide sequences in various biomaterial surfaces have been reported to increase the expression of hepatocellular proteins and biomarkers[13]. Clearly, both topographical features and chemical stimuli afforded by the ECM can be considered independently as important factors that enabled the control and

manipulation cell morphology [14], leading to alterations in migration [15], proliferation [16], and cytoskeleton organization [17].

Several reported research tried to mimic ECM collectively [18] or separately [19] use various types of cues, has been established at the cellular level. These techniques involve the use of co-culture systems [20], polymers [21], recombinant proteins [22] and inorganic compounds [23] and have resulted in the development of functionalized biomaterials that can control cellular behaviors. Of particular interest is the use of TiO_2 deposited thin films, which provide a significant interface for the regulation of hepatocytes attachment and subsequent function [24]. Flexible surface modeling and biofunctionalization, as well as the ability to control thickness, are the main attractions of using these films in tissue engineering research. Consequently, the ability to manipulate a biomaterial to provide a controlled surface texture and chemical composition would allow cellular functions to be increased, as this biomaterial would provide physical and chemical cues to trigger various biological responses.

The role of dimensional and geometrical characteristics of nanofeatures on hepatocellular behaviors has been described in previous chapter in which topography manipulation using EBL and ALD showed an interesting role in the alteration of hepatic functionalities. The utilization of fabricated nanostructures would have several advantages compared to utilizations of natural ECM components (i.e. network of collagen) As the facile and precise control of morphologies and dimensions, plus it avoids the flaws associated with natural origin based material as the presence of biological impurities, or immunogenicity. This urged us to examine the integration of another kind of ECM cues (i.e., chemical cues) with the topography (physical cues), since physical and chemical cues have been proven independently to enhance hepatocellular behaviors. Consequently, such integration between physical and chemical cues will closely mimic the naturally occurring ECM cues and avoid

any drawback associated with utilization of natural based materials. In detail, our aim was to study the collective influence of surface composition and topographical features of substrates on the biofunctionality of a hepatic cell line followed by comparisons between hepatic cellular responses after the utilization of such substrates and other substrates with has only a single cue as topography alone, or RGD alone or even no cues at all (Flat surface or control) are conducted. Little attention has been focused on this to date, even though it is of clear importance for clarifying the relationship between unique ECM cues and *in-vitro* performance of liver cells. Nanogratings with specific dimensional and geometrical characteristics (nanogratings 90 nm wide and 150 nm apart) were fabricated using electron beam lithography and atomic layer deposition. We believe that HepG2 cells recognize these nanopatterns (total dimension 240 nm). As a result of cellular recognition, nanopattern were able induce a change in the cellular behavior with an increase in production of hepatic proteins [25]. These pattern highly mimic the variable diameters of the fibrillar structure of collagen, in which, the individual molecules of triple helical collagen are arranged into fibrils with diameter up to 10 nm, followed by a subsequent arrangement into greater fibrils with a larger diameter (several hundred nm) [26]. Furthermore, the incorporation of organic moieties with specified cellular functionality such as RGD motifs into the TiO_2 inorganic film deposited on surface of nanopattern was accomplished using an enzyme catalyzed oxidation reaction. The morphological and functional changes induced in hepatic cell lines by simultaneously combining the diversified cues were investigated; for example, cellular alignment and the expression of different functional proteins were studied using fluorescent immunostaining techniques.

3. Results

Electron beam lithography (EBL) and atomic layered deposition (ALD) were used to produce dimensionally well-defined nanogratings coated with thin film of TiO_2, and then the RGD peptide was immobilized using an enzyme catalyzed reaction. The effects of simultaneously combining these physical and chemical cues and their influence on HepG2 responses were investigated over a short time course.

Table 3-1: Various substrates used with single or multiple cues to detect their role in the alteration of cellular behaviors.

Code	Physical cues	Chemical cues
(A)		RGD
(B)		-
(C)	Flat	RGD
(D)	Flat	-

3.1 Characterization of substrates with multiple cues

The nanogratings 90 nm wide and 150 nm apart were fabricated by EBL and ALD. The shape, dimensions and topographical features of the fabricated nanopatterns were characterized before and after immobilization of the peptide by atomic force microscope (AFM) (Figure 3-2).

Figure 3-2: 3D AFM images of nanopattern before and after the immobilization of RGD with the fidelity of structures.

The shape and dimensions of the nanogratings were not affected by peptide immobilization, which is important, as the maintenance of topography was a key concern in

this study. Peptide immobilization and surface characteristics were confirmed using time of flight-secondary ion mass spectroscopy (TOF-SIMS) and X-ray photoelectron spectroscopy (XPS) (Figure 3-3A–C). Typical amino acids produced by fragmentation of the peptide (i.e., Glycine, Arginine, Aspartic acid) were assigned by their mass spectral signals and are presented in Figure 3-3A. These characteristic fragments were normalized and compared to the control (The TiO_2 substrate that was subjected to the same procedure without presence of peptide) to determine immobilization intensities between 2 substrates (Figure 3-3B).

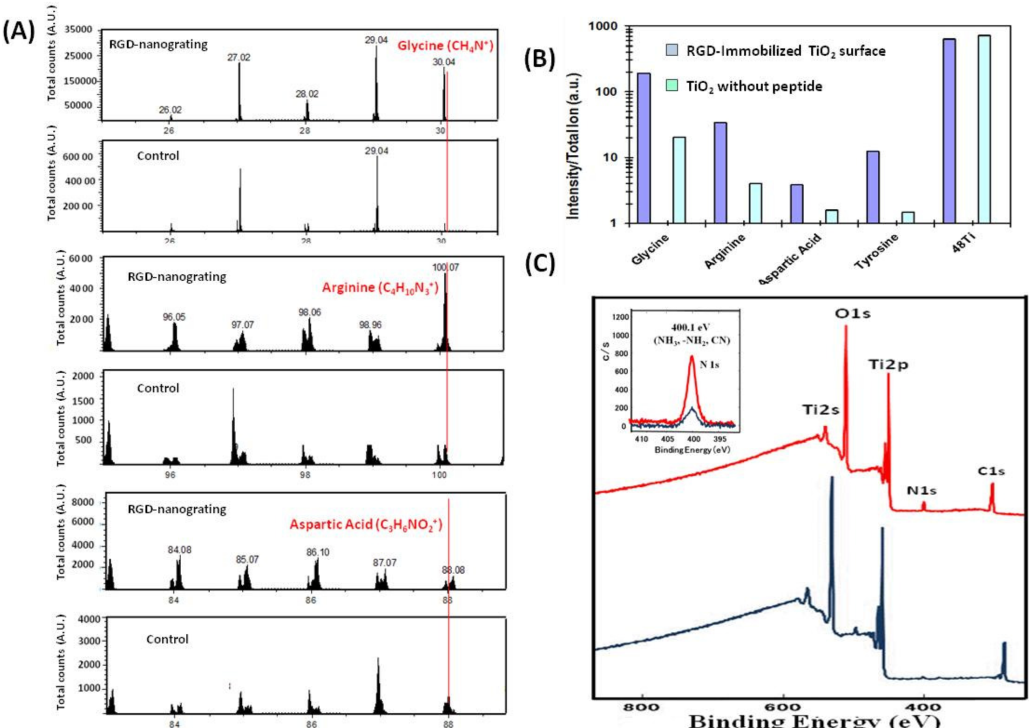


Figure 3-3 (A) Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) confirmed the attachment of RDG peptide on the deposited TiO_2 film, control is a TiO_2 substrate subject to the same immobilization steps without presence of RGD. The specific fragment pattern generated by removal of individual amino acids from peptide is shown in the lower panels. (Glycine *m/z* 30.04 u:CH_4N^+, Arginine *m/z* 100.07 u:$C_4H_{10}N^+$, Aspartic acid *m/z* 88.08 u:$C_3H_6NO_2^+$); (B) Typical peak ratio of amino acids normalized by total ions; (**C**) XPS spectra; wide scan of N1s spectra of TiO_2 coated surfaces before and after the immobilization of peptide. Inset: a high-resolution scan of N1s.

The chemical atomic composition of the RGD-immobilized in TiO_2 coated substrate

was investigated using XPS. Figure 3-3C shows the appearance of N1s signals after peptide immobilization to TiO_2 surfaces, which are absent in the control. High-resolution N1s spectra that confirmed the immobilization of RGD on the TiO_2 substrate is shown in the inset in Figure 3-3C. Thus, these experiments were used to confirm the immobilization of RGD over the nanopattern.

3.2. Influence on functional protein expression

The expression of hepatic functional proteins is stimulated by naturally occurring ECM. Therefore, we examined the integration of physical and chemical factors simulating native ECM to control cellular behaviors. The influence of topography and surface chemistry on cells was determined by investigating substrates that combine both factors.

3.2.1. Alterations in albumin expression

Fluorescence immunostaining of albumin was performed after HepG2 culturing for 12 h, followed by examination of green fluorescent albumin under a fluorescence microscope. Figures 3-4 show fluorescence micrographs of HepG2 cells cultured for 12 h on flat and pattern substrates with and without RGD immobilization. Changes in the chemical composition of the substrate by immobilization of the RGD peptide increased the expression of cytoplasm and nucleus [27] localized albumin compared to substrate without RGD. Furthermore, modulation of topography while maintaining the same surface chemical functionality considerably increased the expression of albumin (green fluorescence).

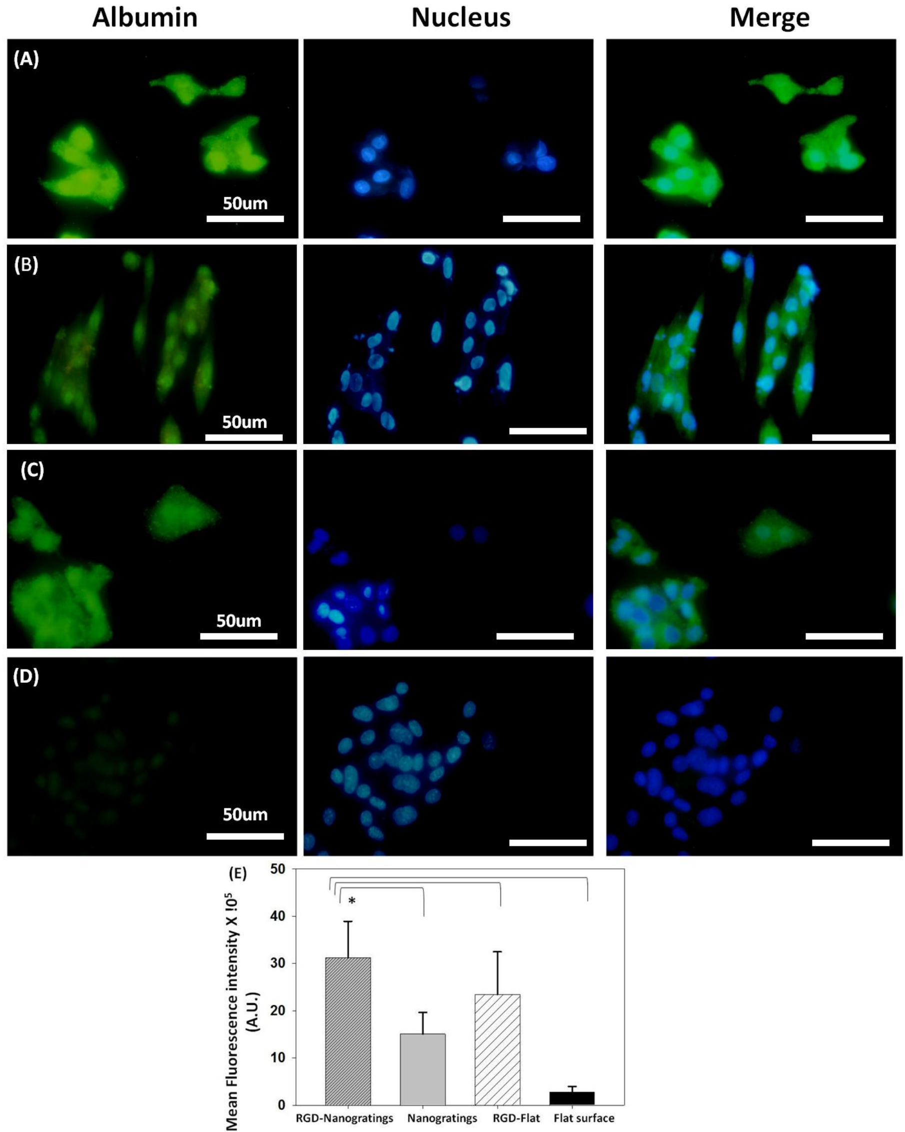


Figure 3-4: Immunofluorescence staining of the hepatic functional protein, albumin (green excitation λ = 448 nm), in HepG2 cells cultured on (A) RGD-nanograting pattern; and (B) nanograting pattern alone; and (C) RGD-flat surface compared to cells cultured on (D) Flat surface alone (control); (E) Relative mean fluorescence intensity calculated using Image J for HepG2 cultured on biofunctionalized substrates *Statistical significance at ($p < 0.05$).

A quantitative comparison of albumin expression is presented in Figure 3-4E, in which we calculated the fluorescence intensities obtained from HepG2 cells cultured our

tested substrates. On which RGD nanogratings showed more than 1.6 fold increases in expression of albumin compared to RGD flat surface, while it showed nearly 2 fold increases compared to nanogratings alone and nearly 10 fold increases compared to flat surface alone. Statistically, the increase in albumin expression was significant based on fluorescence intensity calculations and compared to nanogratings surface alone, an RGD-flat surface, or a flat surface alone ($p < 0.05$). Thus, the results suggest that the 240 nm gratings topography functionalized with RGD from all our tested substrates can be considered to closely mimic the ECM cues essential for the expression of albumin by HepG2 cells.

3.2.2. Alteration in transferrin expression.

The expression of the functional protein transferrin was determined as green fluorescence using an immunostaining technique. Figures 3-5 fluorescence images of transferrin synthesized upon culturing HepG2 on various biofunctionalized substrates. Transferrin expression significantly increased ($p < 0.05$) 12 h after culturing HepG2 on the RGD-nanogratings (Figure 3-5A), while the TiO_2 nanopattern without RGD showed lower transferrin levels (Figure 3-5B). Furthermore, the RGD-flat surface showed a reduction in the expression of transferrin compared to the RGD-pattern substrate (Figure 3-5C). Although transferrin expression is expected in HepG2 cells, the flat surface alone without RGD showed minimal transferrin when compared relatively to the RGD-substrate (Figure 3-5D).

A quantitative comparison of transferrin expression is presented in Figure 3-5E, in which the calculated the fluorescence intensities obtained from HepG2 cells cultured our tested substrates. RGD nanogratings showed more than 1.3 fold increase in expression of transferrin compared to RGD flat surface, while it showed more than 3 fold increase compared to nanogratings alone and more than 4 fold compared to flat surface alone.

Statistically, the increase in transferrin expression was significant based on fluorescence intensity calculations and compared to nanogratings surface alone, an RGD-flat surface, or a flat surface alone ($p < 0.05$).

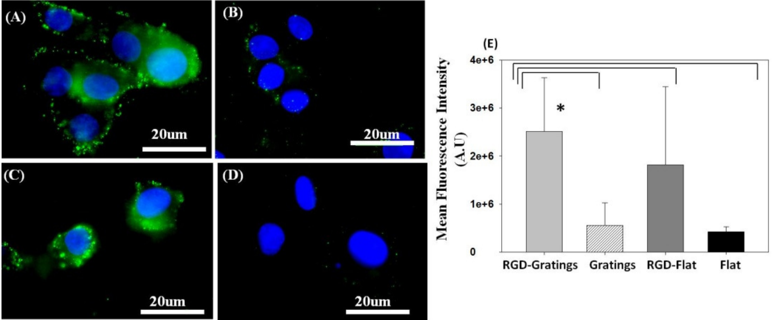


Figure 3-5: Immunofluorescence staining of the hepatic protein, transferrin (green excitation λ= 448 nm), and nucleus (blue) in HepG2 cells cultured on (A) RGD -nanograting pattern; and (B) nanograting pattern compared to cells cultured on (C) RGD-flat surface and (D) Flat alone (control); (E) Relative mean fluorescence intensity calculated using ImageJ for HepG2 cultured on various biofunctionalized substrates. *Statistical significance at ($p < 0.05$).

3.2.3. Alterations in cytochrome P-450 expression

Cytochrome P-450 proteins are a family of hemoproteins found prominently in liver, whose members catalyze the metabolism of a variety of endogenous and xenobiotic substrates [28]. Specifically, cytochrome P-450 2C6 is expressed naturally in the rat liver cells without the presence of specific chemical inducer (i.e., phenobarbital) [29] while its counterpart cytochrome P-450 2C9 is expressed in human liver cells. Thus, the expression of cytochrome P-450 was analyzed after culturing HepG2 cells on RGD- nanopatterned and flat substrates. Figures 3-6 show the formed cytochrome P-450 as red fluorescence inside HepG2 cells cultured on TiO_2 substrates after 18 h. Cells cultured on RGD- nanogratings showed a significant increase in cytochrome P-450 (Figure 3-6A) compared to cells seeded on the nanopatterned, RGD-flat surface, or flat surface (Figure 3-6 B–D).

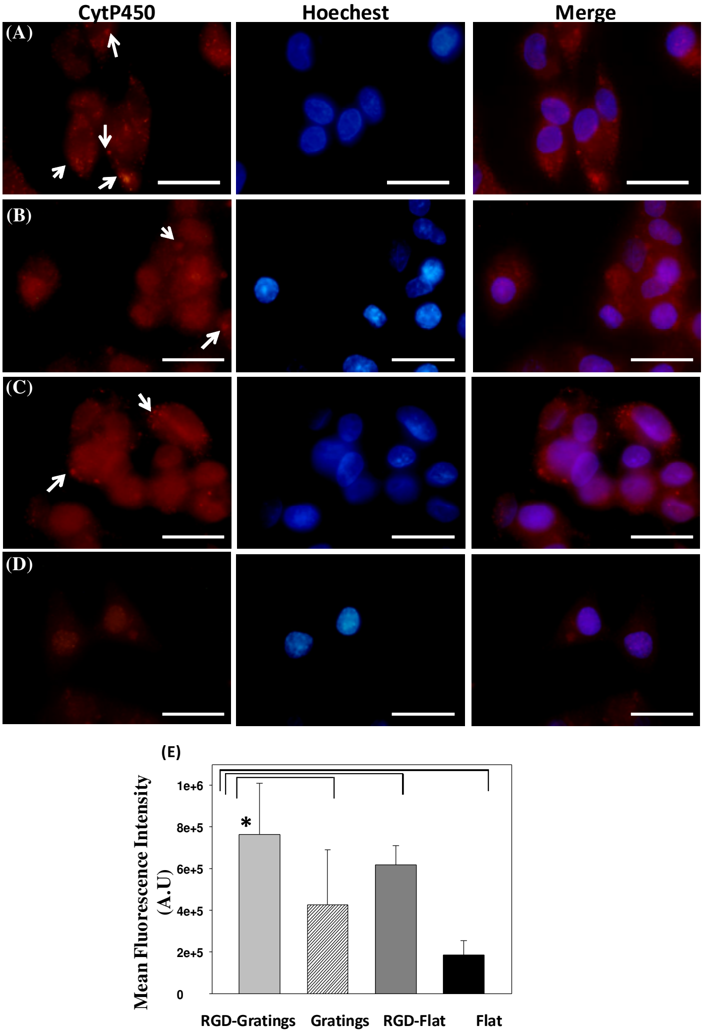


Figure 3-6: Immunofluorescence staining of the hepatic protein, cytochrome P-450 2C9 (red excitation $\lambda = 578$ nm), in HepG2 cells cultured on (A) RGD TiO_2-nanograting pattern; and (B) TiO_2-nanograting pattern; (C) RGD-flat surface compared to cells cultured on (C) and (D) control, arrow heads indicate the fluorescent red dots of cytochrome P-450(Scale bar= 20um); (E) Mean fluorescence intensity calculated using ImageJ for HepG2 cultured on biofunctionalized TiO_2 substrates. *Statistical significance at ($p < 0.05$).

A quantitative comparison of cytochrome P-450 expression is presented in Figure 3-5E, in which we calculated the fluorescence intensities obtained from HepG2 cells cultured our tested substrates. On which RGD nanogratings showed more than 1.2 fold increase in expression of cytochrome P-450 compared to RGD flat surface, while it showed nearly 2 fold

increase compared to nanogratings alone and nearly 4 fold compared to flat surface alone. Statistically, the increase in cytochrome P-450 expression was significant based on fluorescence intensity calculations and compared to nanogratings surface alone, an RGD-flat surface, or a flat surface alone ($p < 0.05$). These results suggest that the manipulation of topography can induce a change in the expression of cytochrome P-450 levels while preserving the same surface chemistry. Furthermore, it indicated the role of chemical cues (RGD) in the stimulation of hepatocellular functionalities.

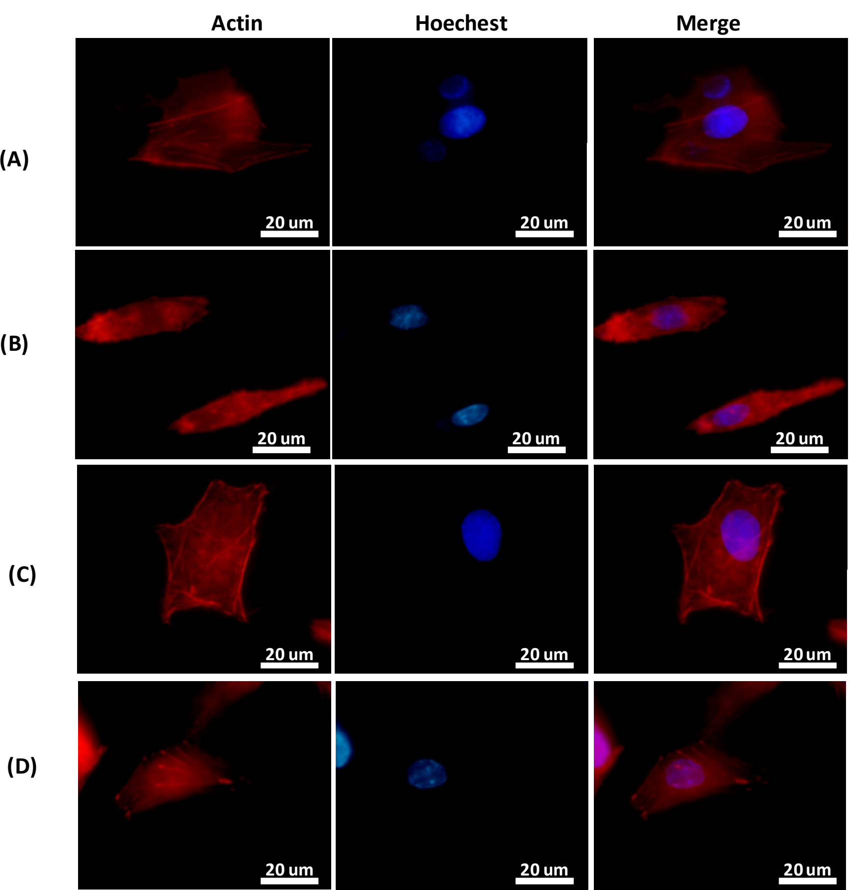


Figure 3-6: Actin fluorescent staining to study the cell alignment and cytoskeletal rearrangement of HepG2 cells cultured for 12 h on (A) RGD- nanogratings compared to (B) nanopattern alone, on which the actin filaments are arranged parallel to the nanofeatures (C) RGD- flat surface and (D) Flat surface alone or control.

3.3. Influence on the structure of hepatic cell line (HepG2)

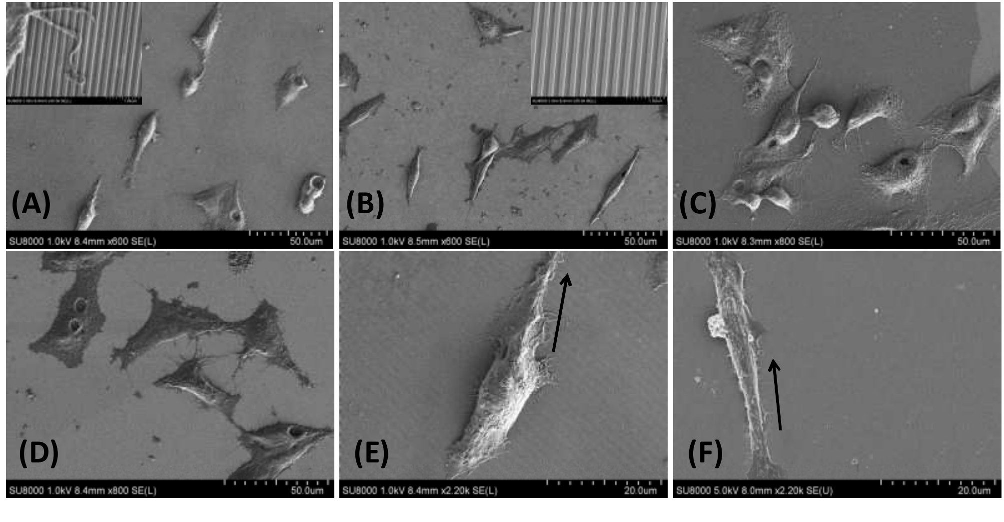
Alterations in topography and chemical surface characteristics could influence cellular orientation, alignment and elongation along nanogratings, in turn regulating cellular behaviors. Thus, actin filament rearrangements and integrin mediated focal adhesions were studied.

3.3.1. Actin filament rearrangement and cell Alignment.

Figure 3-7 shows the fluorescence staining of actin filaments. The staining indicates that the structural reorganization of HepG2 cells to some extent parallels the nanogratings, while less organized cytoskeletons are observed in cell cultures grown on RGD-flat surfaces. Moreover, there was a change in the cellular width when cultured on an RGD-functionalized surface compared to the nanopatterned substrate alone or on a flat surface.

3.3.2. Morphological variations examined using scanning electron microscope

Cellular alignment was investigated using scanning electron microscope. Figure 3-7 shows cellular attachments parallel to the longitudinal axes of TiO_2 gratings while such aligned arrangements cannot be observed in the flat surface with or without RGD. Moreover, the RGD-biofunctionalization of nanopattern resulted in an increase in cellular cytoarchitecture (width and height) when cultured on an RGD-functionalized nanogratings compared to the nanopatterned substrate alone (Figure 3-7E, F), While there is no difference in the percent of aligned cells due to immobilization of RGD on the nanopattern (Figure 3-7 G)



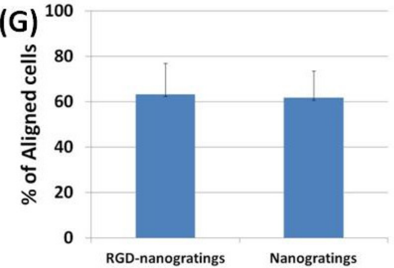


Figure 3-7: SEM images of HepG2 cells cultured for 12 hrs in (A) RGD- nanogratings compared to (B) nanogratings alone and (C) RGD- flat surface (D) flat surface alone (Control), insets are higher magnification SEM images that show the direction of nanogratings and the single cell alignments parallel to nanopattern lines. Furthermore, the increase in cell width and height where indicated in (E) RGD-nanogratings and (F) nanogratings alone while black arrow indicate the direction of nanogratings. (G) No change in the percentage of aligned cells cultured in RGD nanogratings and nanogratings alone.

3.3.3. Integrin mediated focal adhesion

The expression of integrin β1 as green fluorescence upon culturing HepG2 cells on nanogratings with a total dimension of 240 nm and flat surface substrates with and without immobilization of RGD for 12 h is shown in (Figure 3-8). An increase in integrin β1 was observed after culturing HepG2 cells on the RGD immobilized continuous grating compared to cells on the nanopattern alone (Figure 3-8A,B) Thus, the integration of chemical and physical nanocues synergistically facilitates integrin clustering (Figure 3-8A) and further

recruits proteins to focal adhesions. Although the synergistic effects are enhanced compared to each independent nano-cue, individual topographical or chemical nanocues showed nearly comparable stimulation of integrin clustering (Figure 3-8B, C). A quantitative comparison of integrins β1 expression is presented in Figure 3-8E, in which we calculated the fluorescence intensities obtained from HepG2 cells cultured our tested substrates. On which RGD nanogratings showed more than 2 increase in expression of integrin β1compared to flat surface, while it showed nearly 1.5 fold increase compared to nanogratings alone or RGD-flat surface alone. Statistically, the increase in integrin β1 expression was significant based on fluorescence intensity calculations and compared to nanogratings surface alone, an RGD-flat surface, or a flat surface alone ($p < 0.05$).

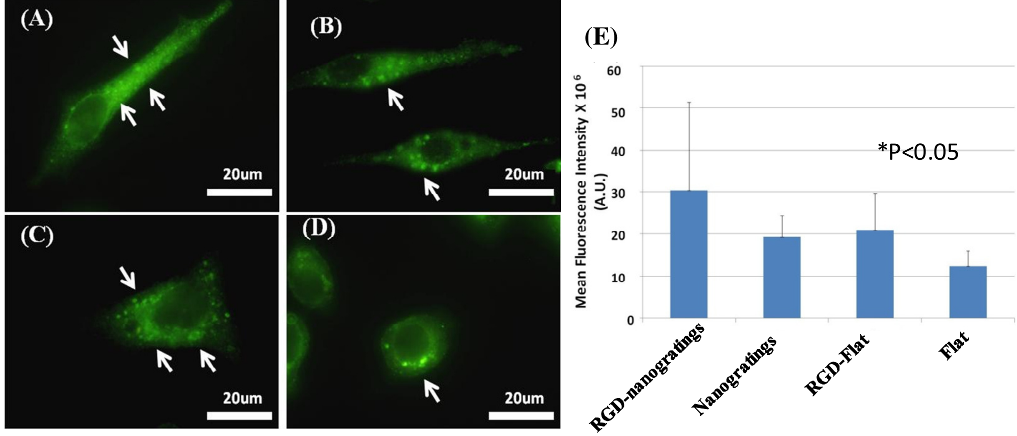


Figure 3-8: An increase in integrin β1 clusters that mediate focal adhesion formation using immunostaining of integrin B1 by Alexa-fluor 488 linked-AB (excitation λ = 488 nm) of HepG2 cells cultured on various substrates on which (A) RGD-nanogratings show high integrin β1 clusters while (B) Nanogratings alone show low integrin B1 clustering compared to it, on the other hand (C) RGD-flat surface shows high integrin β1 clusters, mean while (D) flat substrate alone (control) has the lowest integrin clustering; arrow heads show clusters of integrin B1 as green fluorescent dots. (E) Mean fluorescence intensity calculated using image J for integirn expression after cells cultured for 12 hrs over various pattern and flat substrates with or without RGD.

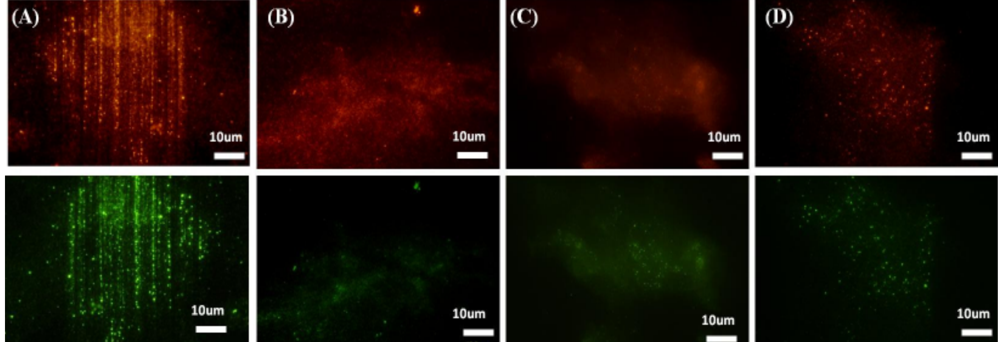


Figure 3-9: ECM components assembled by HepG2 cells cultured for 66 h on various substrates. (A) RGD-nanogratings, (B) nanogratings alone and compared to (C) RGD-flat surface and (D) flat substrate or control alone. Red fluorescence represents collagen IV Ab (2ry-Alexa fluor-540), green fluorescence represents fibronectin (2ry Ab Alexa-fluor-488) after decellularization using H_2O for 1 h at 37 °C.

3.3.4. Alteration in natural extracellular matrix assembly

The influence of nanopattern and RGD immobilization on the expression collagen IV and fibronectin (two naturally assembled ECM components) was studied (Figure 3-9). Decellularization was thus performed and changes in the assembly of fibronectin and collagen IV were examined using an immunostaining technique. Figure 3-9 shows the alteration in the assembly and alignment of native fibronectin and collagen IV after culturing and decellularization of HepG2 cells on an RGD-functionalized nanopatterned surface relative to the nanopatterned substrate alone, RGD-flat surface, and flat surface alone. The assembly of fibronectin and collagen IV was considerably stimulated and aligned in the planar area between the nanogratings on the RGD-functionalized nanopatterned surface compared to other substrates (Figure 3-9A).

4. Experimental & methodology

4.1. Immobilization of RGD on nanopattern substrates

The fabrication of nanopatterned substrate using electron beam lithography and atomic layer deposition was illustrated in chapter 3. The RGD peptide (Proline-Arginine-

Glycine-Asparticacid-Glycine-Glycine-Glycine-Glycine-Glycine-Tyrosine) was purchased from Chinese Peptide Company (Hangzhou, China). The purity of the peptide was 95.9% as determined by high performance liquid chromatography (HPLC) and mass spectrometry. Tyrosinase from mushroom (lyophilized powder, ≥1000 units/mg solid; Sigma-Aldrich, St. Louis, MO, USA) was used for the oxidative reaction. All reagents were dissolved in PBS (diluted 1:10) and sterilized by filtration (0.2 μm Minisart CE, Satorius Stedim, Gottingen, Germany).

The immobilization of peptide on the TiO_2 substrate used an enzyme catalyzed oxidative reaction to link the peptide to the functionalized surface, as reported previously [30]. First, sterilized TiO_2 substrates were immersed in peptide solution (100 μg/mL) and tyrosinase solution (400 μ/mL) was added. The immobilization reaction was carried out for 15 min, and then the peptide-immobilized substrates were removed, washed by immersion in distilled water, and air-dried. nanopatterned substrates before and after RGD immobilization were characterized by atomic force microscopy (SII NanoTechnology L-Trace, Tokyo, Japan) and time of flight-secondary ion mass spectroscopy using a TRIFT V (YLVAC-PHI, Chigasaki, Japan). Fragment patterns generated from amino acids allowed confirmation of the attached surface peptides. The RGD conjugated TiO_2 films coated nanopatterns were characterized by X-ray photoelectron spectroscopy using a PHI Quantera SXM (ULVAC-PHI, Chigasaki, Japan). Fabricated nanopatterned surfaces were characterized using a scanning electron microscope (Hitachi-S3000N, Tokyo, Japan) and atomic force microscopy.

4.2. Hepatic cell culture

HepG2 were cultured in Dulbecco's MEM (Nacalai Tesque, Kyoto, Japan) with 10% heat inactivated FBS and supplemented with 100 U penicillin/100 ug streptomycin (Nacalai Tesque, Kyoto, Japan) per ml medium. All cells were maintained at 37 °C in a 100%

humidified atmosphere under 5% CO_2. At 70%–80% confluency, cells were trypsinized and seeded over the nanopatterned surfaces for 12 or 18 h. Low density cell were used in culture to enable the examination on a almost single cell-basis. For the expression of ECM components, cells were cultured for 66 h with the medium replaced every 33 h. Each experiment was repeated at least three times.

4.3. Immunostaining and fluorescence detection

HepG2 cells cultured on nanopatterned substrates were fixed for 15 min in 4% P-formaldehyde (PFA) in PBS at 4 °C and excess aldehyde was quenched using 0.1 M glycine for 5 min. Cells were permeabilized with 1% triton X-100 in PBS for 5 min, and then primary antibodies were added: rabbit polyclonal anti-albumin antibody-C-terminal diluted 1:25 (Abcam, Cambridge, UK), rabbit polyclonal anti-transferrin antibody diluted 1:500 (Abcam, Cambridge, UK), mouse monoclonal anti-cytochrome P-450 2C6 antibody 1 ug/mL (Abcam, Cambridge, UK), CD29 mouse anti-human mAb-ALexa Fluro® 488 conjugate diluted 1:100 (Life Technologies Invitrogen, Eugene, OR, USA) for integrin B1 at 4 °C overnight. Next, secondary antibodies were added for 1 h: Alexa Fluor® 568 goat anti-mouse IgG (H+L) (Heavy + Light chains) diluted 1:500 (Life Technologies Invitrogen, Euogenes, OR, USA) or goat polyclonal anti-rabbit IgG-H&L-DyLight® 488 diluted 1:500 (Abcam). Observation was performed using an upright fluorescence microscope (Olympus BX51, Tokyo, Japan) equipped with an Olympus DP70 digital camera. DP Controller Ver. 2.1.1, (Olympus corporation, Tokyo, Japan) was used to process the images. Quantitative comparison of albumin expression was determined using the freeware image analysis software, Image J, WS Rasband, National Health Institute, Bethesda, MA, USA) as previously reported [31]. Cell area was determined by manual delineation of raw fluorescence images. A minimum of 12 cells were analyzed from two independent experiments.

Cytoskeletal F-actin was visualized by treating the cells with phalloidin-TRIC and Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) at 5 µg/mL for 15 min were used.

To investigate ECM components, decellularization was performed as previously reported [32] on cells cultured on biofunctionalized nanopatterned and flat surfaces for 66 h, followed by incubation with 2 mL of distilled water for 1 h at 37 °C. Various surfaces were washed by immersion in PBS. The deposited ECM was fixed using 4% PFA for 20 min, followed by permeabilization with 0.5% triton X-100 in PFA. Primary antibodies such as mouse monoclonal antifibronectin diluted 1:50 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and mouse monoclonal anticollagen IV diluted 1:50 (Santa Cruz Biotechnology Inc.) were added for 30 min, and then secondary antibodies (goat anti-mouse IgG-TR diluted 1:250 (Santa Cruz Biotechnology Inc.), Alexa Fluor® 488 goat anti-mouse IgM (H+L) diluted 1:250 (Life Technologies Invitrogen, Euogenes, OR, USA) were added for 30 min. The effective removal of cells and cellular debris by this method was verified by fluorescence labelling of the decellularized surfaces using Hoechst 33342.

4.4. Scanning electron microscope investigation

Investigations of the cell rearrangements and alignments were done by scanning electron microscopy (SEM) using (Hitachi S-4800, Japan). Prior to SEM investigations the samples were prepared as follows. At the end of the culture period the cells on the substrates were fixed with 2.5 vol% glutaraldehyde (Wako, Japan) in PBS for 2 h. After which the cells were dehydrated through a series of ethanol concentrations (10%, 40%, 60%, 80%, 100%) for 5 min, respectively. Final desiccation was done using freeze drying (Hitachi Es 2030, Tokyo, Japan). Finally, the samples were placed on SEM specimen holders and observed under field-emission scanning electron microscope operated at an acceleration voltage in the range 1–5 kV.

5. Discussion

We examined the integration of chemical and physical cues to study the influence of such integration on the enhancement of hepatic cellular behavior. These assembled constructs mimic the native environment around cells. We used HepG2 cells, as they exhibit a lower cellular functionality upon culturing under flat tissue culture conditions [33] moreover, HepG2 is a stable cell line compared to primary hepatocytes, consequently the drawbacks associated with the alteration in the primary hepatocytes' native culture environments upon their culture into a rigid flat substrate is avoided. Such alterations would result in a subsequent change in cell morphology, polarity and gene expression [34], which may cause discrepancies in results.

Alterations in the physicochemical characteristics of a biomaterial impact the modulation of cellular behaviors [35–39] since surface chemistry [12] and substratum topography [40-41] each have profound effects. We examined the effect of manipulating physical cues on hepatic cellular behaviors and showed that alterations in the shape or dimensional characteristics of physical cues critically affect hepatocellular function [25]. These alterations mimicked the physical cues of naturally occurring ECM components to control and regulate multiple hepatic functions (Chapter 2). Here, we investigated the combined impact of diverse cues on hepatocellular behaviors. The RGD peptide was immobilized on nanopatterns that mimic the hierarchically extended collagen nanofibrillar structure and hepatocellular behaviors were investigated. At first, cell viability upon culturing on TiO_2 pattern and flat substrates was examined. The viability investigation was done using live/dead staining for intracellular esterase activity and cell membrane integrity. The use of pattern substrates showed no change in the viability (~98%) compared to flat surface.

An increase in the expression of the important functional proteins albumin, transferrin,

and cytochrome P-450 was determined. A significant enhancement in expression of these liver-specific markers on RGD coated surfaces was observed compared to uncoated substrates, regardless of the topography of the substrate. This could be due to the activation of an integrin-dependent intracellular signaling pathway. Alterations in the surface chemical composition of biomaterials modulate charge, hydrophilicity, and protein adsorption, with subsequent alteration in cellular affinity for the substrate [42, 43]. The substrate that integrated nanotopography with the adhesive peptide motif maximally increased most hepatocellular functionalities tested. The synergistic effect of combining both chemical and physical cues provides the cell integrity and orientation, with a subsequent activation of specific cell membrane integrins. Thus, the better the ECM intrinsic elements are mimicked, the better the substrate's cytocompatibility and the functionality of HepG2. Less of an effect was observed for transferrin expression, as hepatic cell functionality mainly depends on intricate signals and pathways that are activated by diversified cues. The detailed mechanisms underlying such synergism, and identification of their essential regulating factors, require further study. However, we can conclude that chemical cues play a major role in the enhancement of hepatic functionality compared to substratum topography alone, and the integration of both cues has a synergistic effect on most hepatic functionalities. Thereby, our findings suggest that surface nanofeatures with well-defined dimensions and chemical composition that imitate ECM fibrillar structures and containing a specific motif (e.g., RGD) could be utilized to increase multiple cell functions, rather than simply using nanotopography or chemical cues alone. Since the pattern and flat areas exist in the same substrate, it is difficult to separate cells; further quantification such as RT-PCR or ELISA, were therefore not accurate to perform.

Since cell morphology and alignment mainly rely on the surface characteristics of the

biomaterial, the concurrent effect of immobilization of RGD on the cytoskeleton, actin filament assembly and reorganization were observed. HepG2 cells cultured on dimensionally well-defined nanograting substrates or flat surfaces with and without RGD biofunctionalization were examined using fluorescence staining and SEM. RGD-immobilized substrates resulted in an increase in cell width and height, and a decrease in cell extension (length) compared to uncoated substrates, suggesting enhanced cell-substratum affinity. These alterations including morphology and alignments may be mediated by the presence of adhesive ligands that encourage the organization of three dimensional actin cytoskeletons; actin filament reorganization is mediated by the assembly of intracellular and extracellular complex domains (integrins) that bridge adhesive ligands (RGD) and the actin cytoskeleton. Furthermore, this was confirmed by SEM, since integration of both surface chemistry and nanotopography resulted in a change in the cellular morphology compared to nanogratings alone. While the % of single cells aligned along the long axis of the underlying topographical features have not been changed before and after the immobilization of RGD. These observations suggest that the presence of chemical cues such as the RGD motif result in increased substrate affinity and play a dominant role in cellular morphology, as do physical nanocues such as topography. Thus, the presence of discrete surface cues is essential for selective cell adhesion and activation, indicating that these cues have clear applications in regenerative medicine.

Integrin β1 mediated focal adhesion was compared on the nanograting surface and the flat surface without or with RGD using immunofluorescence staining of a transmembrane protein, integrin β1. Integrin-mediated signals are leading mediators for controlling cellular behavior, adhesion, and functionality. Cellular behavior is dependent on integrin interactions with the substratum: the integrin extracellular domains bind to a specific motif on the ECM,

and their intracellular domains are associated with the actin cytoskeleton and affiliated proteins. Biochemical motifs, as well as topographical characteristics, such as dimension, shape, symmetry, and roughness separately play dominant roles in integrin clustering and focal adhesion formation [44-46].

Regarding the role of mimicking the ECM cues for the assembly of native ECM, RGD alone, acting as a chemical cue, enhanced cell-substratum affinity, resulting in the up-regulation of the expression of native assembled ECM components. The presence of nanogratings that resemble collagen fibrils acted as vertical ledges in between the planar areas. This topography increased the assembly of ECM components and aligned them in between these ledges. Although integration of RGD with topography showed aligned structures while other substrates showed random structures, the dimensions and size of assembled proteins cannot be recognized from such experiments. Consequently, the collective role of physical and chemical cues profoundly altered the active assembly of ECM components, resulting in further modification in tissue reorganization.

6. Conclusions

The collective role of chemical (RGD) and physical (topography) cues on the behavior of hepatic cells was studied and compared with the role of each cues separately or without any cues. Substrates that simultaneously integrated nanotopography with an adhesive peptide motif (RGD) maximally increased hepatocellular functionality. A significant enhancement in the expression of liver-specific markers was observed on RGD-coated surfaces compared to uncoated substrates. These results emphasize the major role of chemical cues in enhancing hepatic functionality compared to substratum topography alone. The manipulation of chemical cues and/or distinct topographical features caused modifications in cellular

reorganization mediated by changes in cellular attachments, spreading, integrin clustering and assembly of ECM components. Thus, we conclude that modifications in the physicochemical characteristics of biomaterials (Topography and RGD), either independently or by combining both chemical and physical cues influence the cell functionalities and architecture, with subsequent alterations in the behavior of hepatic cells. These findings could be utilized in the design of bio-inspired functionalized constructs for hepatic bioreactors and other applications.

7. References

[1] Engel J, Chiquet M, An over view of extracellular matrix and function. In The Extracellular Matrix: An Over View; Mecham RP, Ed.; Springer: Berlin/Heidelberg, Germany, 2011; pp. 1–41.

[2] Lukashev ME, Werb Z, ECM signalling: Orchestrating cell behavior and misbehavior, Trends Cell Biol., 1998; 8: 437–441.

[3] Pamula E, de Cupere V, Dufrene YF, Rouxhet PG, Nanoscale organization of adsorbed collagen: Influence of substrate hydrophobicity and adsorption time, J Colloid Interface Sci., 2004; 271: 80–91.

[4] Bozec L, van der Heijden G, Horton M, Collagen fibrils: Nanoscale ropes, Biophys J., 2007; 92: 70–75.

[5] Vaday GG, Lider O, Extracellular matrix moieties, cytokines, and enzymes: Dynamic effects on immune cell behavior and inflammation, J Leukocyte Bio., 2000; 67: 149–159.

[6] Bosman FT, Stamenkovic I, Functional structure and composition of the extracellular matrix, J Pathol., 2003; 200: 423–428.

[7] Zeisberg M, Maeshima Y, Mosterman B, Kalluri R, Renal fibrosis. Extracellular matrix microenvironment regulates migratory behavior of activated tubular epithelial cells, Am. J Pathol., 2002; 160: 2001–2008.

[8] Heino J, The collagen receptor integrins have distinct ligand recognition and signaling functions, Matrix Biol., 2000; 19: 319–323.

[9] Ahmed N, Riley C, Rice G, Quinn M, Role of integrin receptors for fibronectin, collagen and laminin in the regulation of ovarian carcinoma functions in response to a matrix microenvironment, Clin Exp Metastasis., 2005; 22: 391–402.

[10] Biggs MJP, Ritchard RG, Dalby MJ, Nanotopographical modification: A regulator of cellular function through focal adhesions, Nanomed: Nanotechnol Biol Med., 2010; 6: 619–633.

[11] Wozniak MA, Modzelewska K, Kwong L, Keely PJ, Focal adhesion regulation of cell behavior, Biochim Biophys Acta., 2004; 1692: 103–119.

[12] Maheshwari G, Brown G, Lauffenburger DA, Wells A, Griffith LG, Cell adhesion and motility depend on nanoscale RGD clustering, J Cell Sci., 2000; 113: 1677–1686.

[13] [34] Bartolo LD, Morelli S, Lopez LC, Giorno L, Campana C, Salerno S, Rende M, Favia P, Detomaso L, Gristina R, d’ Agostino R, Drioli E,Biotransformation and liver-specific functions of human hepatocytes in culture on RGD-immobilized plasma-processed membranes. Biomaterials, 2005; 26: 4432-4441.

[14] Wang N, Ingber DE, Control of cytoskeletal mechanics by extracellular cell shape, and mechanical tension, Biophys J., 1994; 66: 2181–2189.

[15] Abrams GA, Goodman SL, Nealy PF, Franco M, Murphy CJ, Nanoscale topography of the basement membrane underlying the corneal epithelium of the rhesus macaque, Cell Tissue Res., 2000; 299: 39–46.

[16] Hornberger LK, Singhroy S, Cavalle-Garrido, T, Tsang W, Keeley F, Rabinovitch M, Synthesis of extracellular matrix and adhesion through β1 integrins are critical for fetal ventricular myocyte proliferation, Circulation Res., 2000; 87: 508–515.

[17] Wu C, Dedhar S, Integrin-linked kinase (ILK) and its interactors: A new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes, J Cell Biol., 2001; 155: 505–510.

[18] Wang P-Y, Wu T-H, Chao P-HG, Kuo W-H, Wang M-J, Hsu C-C, Tsai W-B, Modulation of cell attachment and collagen production of anterior cruciate ligament

cells via submicron grooves/ridges structures with different cell affinity, Biotechnol Bioeng., 2013; 110: 327–337.

[19] Kong YP, Tu C, Donovan PJ, Yee AF, Expression of Oct4 in human embryonic stem cells is dependant on nanotopographical configuration, Acta Biomater., 2013; 9: 6369–6380.

[20] Takayama G, Taniguchi A, Okano T, Identification of Differentiall expressed genes in hepatocyte/endothelial cell co-culture system, Tissue Eng., 2007; 13: 159–166.

[21] Bierwolf J, Lutgehetmann M, Feng K, Erbes J, Deichmann S, Toronyi E, Stieglitz C, Nashan B, Ma PX, Pollok JM, Primary rat hepatocyte culture on 3D nanofibrous polymer scaffolds for toxicology and pharmaceutical research, Biotech Bioeng., 2011; 108: 141-150.

[22] Nishida, Y, Taniguchi, A. Induction of albumin expression in HepG2 cells using immobilized simplified recombinant fibronectin protein, In-vitro Cell Dev Biol-Anim., 2013; 49: 400–407.

[23] Hoessa A, Teuschera N, Thormanna A, Aurichb H, Heilmanna A, Cultivation Of Hepatoma Cell Line HepG2 On Nanoporous Aluminum Oxide Membranes, Acta Biomater., 2007; 3: 43–50.

[24] Nakazawa K, Lee S-W, Fukuda J, Yang D-H, Kunitake T, Hepatocyte spheroid formation on a titanium dioxide gel surface and hepatocyte long-term culture, J Mater Sci., 2006; 17: 359–364.

[25] Abdellatef SA, Ohi A, Nabatame T, Taniguchi A, Induction of hepatocyte functional protein expression by submicron/nano-patterning substrates to mimic *in-vivo* structures, Biomater Sci., 2014; 2: 330–338.

[26] Prockop DJ, Fertala A, The collagen fibril: The almost crystalline structure, J Struct Biol., 1998; 122: 111–118.

[27] Hamashima Y, Harter JG, Coons AH, The Localization of Albumin and fibrinogen in human liver cells, J Cell Biol., 1964; 20: 271–279.

[28] Kocarek TA, Schuetz EG, Guzelian PS, Expression of multiple forms of cytochrome p450 mrnas in primary cultures of rat hepatocytes maintained on matrigel, Mol Pharmacol., 1993; 43: 328–334.

[29] Shaw PM, Weiss MC, Adesnik M, hepatocyte nuclear factor 3 is a major determinant of cyp2c6 promoter activity in hepatoma cells, Mol Pharmacol., 1994; 46: 79–87.

[30] Park KM, Park KD, Facile surface Immobilization of cell adhesive peptide onto TiO_2 substrate via tyrosinase-catalyzed oxidative reaction, J Mater Chem., 2011; 21: 15906–15908.

[31] Burgess A, Vigneron S, Brioudes E, Labbé J-C, Lorca T, Castro A, Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance, Proc Natl Acad Sci., 2010; 107: 12564–12569.

[32] Herrema H, Czajkowska D, Théard D, van der Wouden JM, Kalicharan D, Zolghadr B, Hoekstra D, van Ijzendoorn SCD, Rho Kinase, myosin-ii, and p42/44 mapk control extracellular matrix-mediated apical bile canalicular lumen morphogenesis in HepG2 cells, Mol Biol Cell., 2006; 17: 3291–3303.

[33] Bokhari M, Carnachan RJ, Cameron NR, Przyborski SA, Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function Culture of HepG2 liver cells on polystyrene scaffolds enhance the cell structure and function during toxicological challenge, J Anat., 2007; 211: 567–576.

[34] Soldatow VY, LeCluyse EL, Griffth LG, Rusyn I, In-vitro models for liver toxicity testing, Toxicol Res., 2013; 2: 23–39.

[35] Zhu B, Lu Q, Yin J, Hu J, Wang Z, Alignment of osteoblast-like cells and cell-produced collagen matrix induced by nanogrooves, Tissue Eng., 2005; 11: 825–834.

[36] Watari S, Hayashi K, Wood JA, Russell P, Nealey PF, Murphy CJ, Genetos DC, Modulation of osteogenic differentiation in hMSCs cells by submicron topographically-patterned ridges and grooves, Biomaterials, 2012; 33: 128–136.

[37] Kulangara K, Yang Y, Yang J, Leong KW, Nanotopography as modulator of human mesenchymal stem cells function, Biomaterials, 2012; 33: 4998–5003.

[38] Biela SA, Su Y, Spatz JP, Kemkemer R, Different sensitivity of human endothelial cells, smooth muscle cells and fibroblasts to topography in the nano-micro range, Acta Biomater., 2009; 5: 2460–2466.

[39] Choi C-H, Hagvall SH, Wu BM, Dunn JCY, Beygui RE, Kim C-J, Cell interaction with three-dimensional sharp-tip nanotopography, Biomaterials, 2007; 28: 1672–1679.

[40] Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CDW, Oreffo ROC, The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder, Nat Mater., 2007; 6: 997–1003.

[41] You M-H, Kwak MK, Kim D-H, Kim K, Levchenko, A, Kim D-Y, Suh K-Y, Synergistically Enhanced osteogenic differentiation of human mesenchymal stem cells by culture on nanostructured surfaces with induction media, Biomacromolecules, 2010; 11: 1856–1862.

[42] Cai K, Frant M, Bossert J, Hildebrand G, Liefeith K, Jandt KD, Surface functionalized titanium thin films: Zeta-potential, protein adsorption and cell proliferation, Colloids Surf B., 2006; 50: 1–8.

[43] Porté-Durrieu MC, Guillemot F, Pallu S, Labrugère C, Brouillaudc B, Bareille R, Amédée J, Barthe N, Dard M, Baquey C, Cyclo-(DfKRG) peptide grafting onto ti-6al-4v: physical characterization and interest towards human osteoprogenitor cells adhesion, Biomaterials, 2004; 25: 4837–4846.

[44] Lange R, Lüthen F, Beck U, Rychly J, Baumann A, Nebe B, Cell-extracellular matrix interaction and physico-chemical characteristics of titanium surfaces depend on the roughness of the material, Biomol Eng., 2002; 19: 255–261.

[45] Curtis AS, Casey B, Gallagher JO, Pasqui D, Wood MA, Wilkinson CD, Substratum nanotopography and the adhesion of biological cells. Are symmetry or regularity of nanotopography important?, Biophys Chem., 2001; 94: 275–283.

[46] Sato M, Aslani A, Sambito MA, Kalkhoran NM, Slamovich EB, Webster TJ, Nanocrystalline hydroxyapatite/titania coatings on titanium improves osteoblast adhesion, J Biomed Mater Res A., 2008; 84: 265–272.

Chapter 4

Application: Improvement of the sensitivity for drug cytotoxicity analysis using nanopattern that mimics ECM as a culture substrate.

1. Summary

Here, we tried to develop a more sensitive drug toxicity analysis model by the utilization of nanopattern that mimics ECM cues as a culture substrate. Since the role of ECM simulation using nanopattern in the variation of hepatic cell line cellular response to a cytotoxic agent “cisplatin’ was investigated. The presence of such topography induced a change in the cellular response to various concentration of cisplatin. At beginning, we studied the cell-substratum induced variation of cisplatin cytotoxicity; a higher cytotoxic response to cisplatin was observed for cells cultured on the nanopattern relative to a flat surface. Moreover, the nanofeatures that mimic ECM in the presence of cisplatin induced changes in the chromatin condensation, shape, cellular orientation, and alignment compared to flat surface, as these nanostructures fabricated by electron beam lithography and atomic layer depositions with well defined dimensions (240 nm in size) and longitudinal extended geometry (nanogratings) were able to be recognized by such cells even in cytotoxic conditions. Accordingly, such results emphasize the role of topography as an important player in the cellular response to cisplatin. Therefore, the developments of biomimetic substrates with topography that simulate ECM cues could have potentials in the drug development strategies.

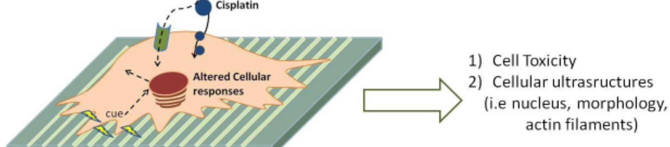


Figure 4-1: Alteration of cellular behaviors of HepG2 cells cultured over the nanopattern in the presence of cisplatin.

2. Background

Extracellular matrix (ECM) is the cellular native environment, and is composed of organic matter and macromolecules that are naturally produced and assembled into three-dimensional meshworks with precisely contoured nanostructures. ECM is composed of several proteins such as collagens, laminins, fibronectin, glycosaminoglycans, and proteoglycans. The individual ECM molecules, such as collagen moieties, which are fibrous proteins with a triple helical structure, are approximately 300 nm long and 1.5 nm wide [1]. These helices are assembled into nanofibrillar networks that hierarchically extend for tens of micrometers in length and have diameters (30-400 nm) with periodical cross-striations [2]. The architecture of ECM possesses essential physical cues and biochemical factors that trigger required cellular behaviors [3-5].

Consequently, mimic such ECM cues by the manipulation of biomaterial characteristics (i.e. topography, stiffness, roughness, surface chemistry...ect.) would participate in generation of bio-specialized systems for the drug development research [6] and tissue engineering applications [7]. For cancer cells likewise normal cells any change in the native ECM characteristics as composition or biophysical characteristics will be recognized and transduced by cells through receptors as integrins (transmembrane proteins) into specific signaling pathways and provoke a variety of cellular cascades and events [8]. These cues (chemical or physical) in addition to signaling factors give rise to the tumor microenvironment, which is responsible for continuous alteration in tumor growth, progression, invasion, transformation, and even drug resistance [9-12]. For instance, disruptions in the structural integrity of the epithelial ECM (basement membrane) have been associated with the disorganization of pancreatic carcinoma [13, 14]. Furthermore, restoration of morphologically normal cellular structures or the death of tumor cells results

when several types of breast cancer cell are cultured in 3D reconstituted basement membrane in the presence of specific inhibitors that block the signaling pathways stimulated in these specific phenotypes [14]. Consequently, several studies have been performed to clarify the relationship between mimiced ECM cues and cancer cells. For instance, ECM compositions are of interest, since the manipulation of ECM proteins is accompanied by an alteration in chemotherapeutic sensitivity [15, 16]. Another factor is the role of matrix stiffness in the alteration of cancer cell characteristics. Stiffer artificial material is able to increase therapeutic resistance with lower clongenic capacity, while softer material induces reversible cellular dormancy in hepatic cell lines [17]. For natural ECM, a decrease in stiffness by altering collagen crosslinking delays malignant growth and tumor development [18]. Moreover, the use of 3-D porous polystyrene supports as a substrate for cell culture results in less susceptibility to methotrexate cytotoxicity compared to 2-D cultures [19]. Furthermore, the use of 3D scaffolds fabricated from chitosan-alginate is correlated with an increase in the malignancy and invasion of different glioma cells with an increase in the production of mediators responsible for angiogenesis [20].

However, some of these reported results are contradictory, and they provide insufficient evidence to establish an explicit relationship between single ECM elemental cues and cancer development, responses and therapeutic resistance. Such relationships mainly depend on the cellular model used in addition to complicated networks of overlapping cellular cascades. Furthermore, little attention has been focused on nanotopography especially that mimic ECM cues and cancer cell responses to cytotoxic agents, even though it is essential for the development of biomaterials that could be utilized in the more reliable drug development strategies.

In the present study, alterations in the cellular response of a hepatic cell line under

cytotoxic conditions was determined, while nanopattern (240 nm) that mimics ECM cues, in particular the fibrillar structure of collagen, was used as a culture substrate. This substrate previously showed improved cytocompatibility and hepatocellular functionality [21]. Our main goal was to compare cellular changes due to the presence of nanostructures after exposure to the cytotoxic agent cisplatin. Cisplatin is one of most effective and widely used cytotoxic agents for treatment of several types of cancers, it has a great affinity toward nucleophilic centres of biomolecules since it forms bifunctional adducts with such molecules as DNA. Despite its prevalent utilization in many kinds of cancer therapy, the continually emerging resistance is a major obstacle for such utilization. Furthermore, its utilization for the cancer therapy is associated with several side effects and other organ toxicities as hepatic toxicity [22]. Therefore, mimic the native cellular environment or ECM cues to further understand the relationship between cell responses and such drug is substantial especially in condition that closely mimic ECM. Here, we investigated alterations in cellular cytotoxicity and morphology induced by the coexistence of these factors using fluorescent microscopy and scanning electron microscopy (SEM). We showed that the presence of these nanostructures was capable of affecting the hepatic cellular response. These results are important for understanding the relationships between ECM biophysical cues, chemotherapeutics, and anti-cancer drug responses and associated side effects.

3. Results

In this study, the role of nanotopography as a major determinant of ECM and its manipulation of hepatocellular cytotoxic responses were determined in the presence of cisplatin. We investigated the influence of nanopattern that mimics ECM cues in the alteration of cellular behaviors in the presence of the drug. As these assembled constructs

mimic the native environment around the cells, we then studied the cell-substratum-induced variation in cytotoxicity caused by cisplatin over a short time of exposure, 12 hrs. Such nanopattern was fabricated using electron beam lithography and atomic layer deposition. The shape and dimension of fabricated nanopattern are illustrated using SEM and AFM (as discussed in Chapter 3).

3.1. Alteration in cisplatin cytotoxic responses

To understand some of the discrepancies in the behavior of cytotoxic agents *in-vivo* and *in-vitro*, we examined the role of nanotopography on cytotoxic responses. An increase in the percentage of dead cells was found after culturing HepG2 cells over the nanopattern compared to a flat substrate after exposure to various cisplatin concentrations for 12 hrs by fluorescence microscopy. Figure 4-2 shows fluorescent micrographs of dead (red) and alive (green) HepG2 cells grown on nanopattern/flat substrates in the presence of three different concentrations of cisplatin. The modulation of topography significantly influenced the cytotoxicity induced by cisplatin (red fluorescent cells). In general, the biocompatibility of such substrate was previously reported [21], as there is no change in viability for cells cultured on the substrates alone without any cytotoxic agent. However, after cisplatin exposure, a higher cytotoxic response was observed on the nanopattern relative to the flat surface (Figure 4-2a). A significant increase in the percentages of dead cells (almost 2 fold increase) was observed upon the change of topography, especially in the 0.25 and 0.5 mM cisplatin concentrations, relative to the flat surface ($P < 0.05$) (Figure 4-2b). While the highest cisplatin concentration (0.75 mM) showed an increase in the percentage of dead cells, it was not statistically significant compared to the flat surface.

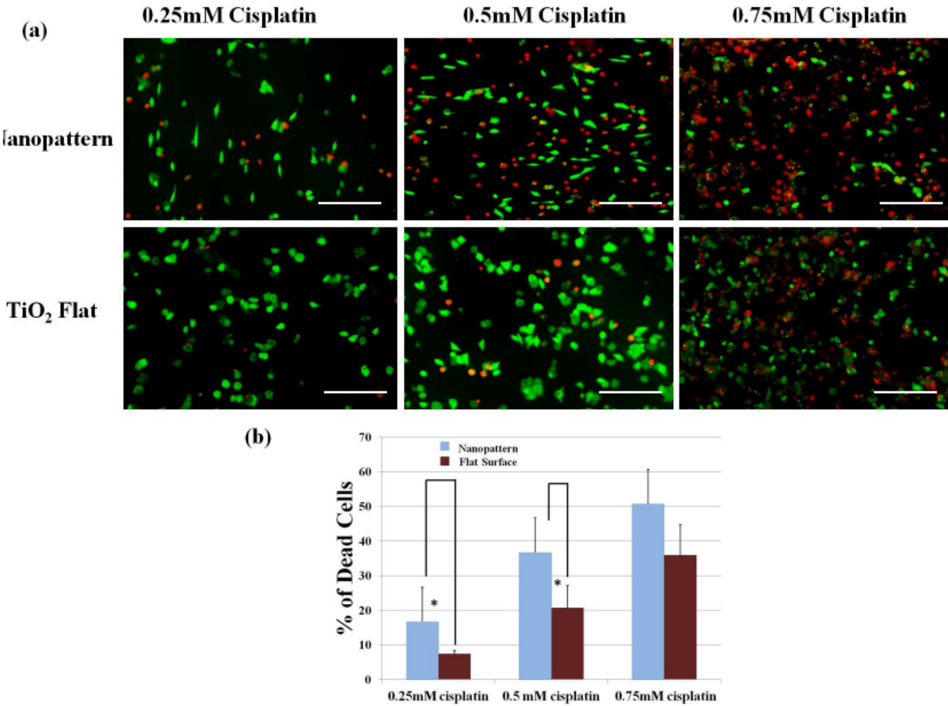


Figure 4-2: (a) Live/dead fluorescence staining of HepG2 cells cultured o the nanopattern (upper panel) and flat surface (lower panels).on which green cells are living and red cells are dead. (b) Average percentages of dead cells ($n \geq 5$) upon culturing HepG2 on substrates with various cisplatin concentrations. *: statistically significant at $P<0.05$. Scale bar = 200 µm.

3.2. Role of CTR-1 expression

We investigated the level of CTR-1 expression using immunofluorescent techniques. Figure 4-3 shows fluorescent images of CTR-1 upon culturing of HepG2 on nanopattern and flat substrates. The expression CTR-1 has almost 1.3 fold increase after culturing of HepG2 on the 240 nm nanopattern with a linear nanopattern (Figure 4-3A upper panel) for 20 hrs compared to cells cultured on flat surface (Figure 4-3A lower panel). Such different was statistically significant when compared using calculated fluorescent intensities (Figure 4-3B).

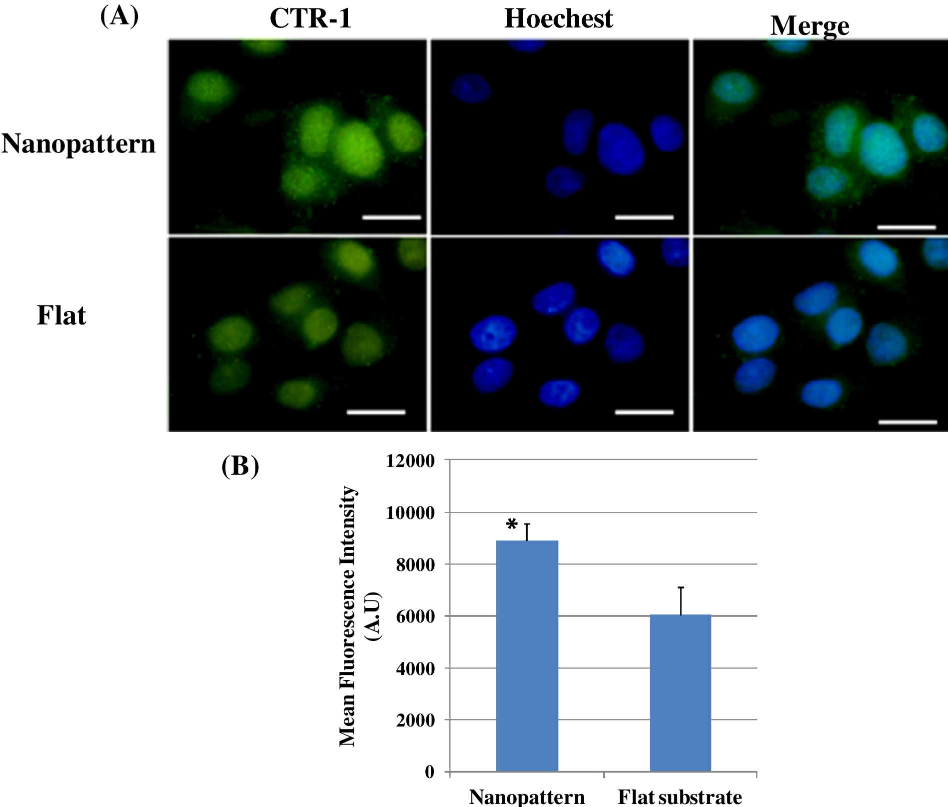


Figure 4-3: (A) Immunofluorescent staining of copper transporter-1 (green excitation λ = 448 nm) in HepG2 cells cultured on nanopattern with total dimensions and grating shapes of 240 nm (Upper panel) and flat surface (lower panel) (Scale bar = 20um). (B) Calculated mean fluorescence intensity than shows significant fluorescence intensity in cells cultured over nanopattern compared to flat surface. * Statistically significant at ($p < 0.05$).

3.3. Hepatocellular nuclear and morphological variations

The presence of cisplatin in general induces specific cellular and morphological variations. Meanwhile, altered substratum topography alone induces transformations in shape, cellular orientation, cell alignment, and elongation. Therefore, we examined these cellular variations induced by cisplatin in presence of the biomimetic construct. Thus, altered nuclear chromatin condensation, morphological variability, and directional modifications were examined by different techniques such as fluorescent microscopy and SEM.

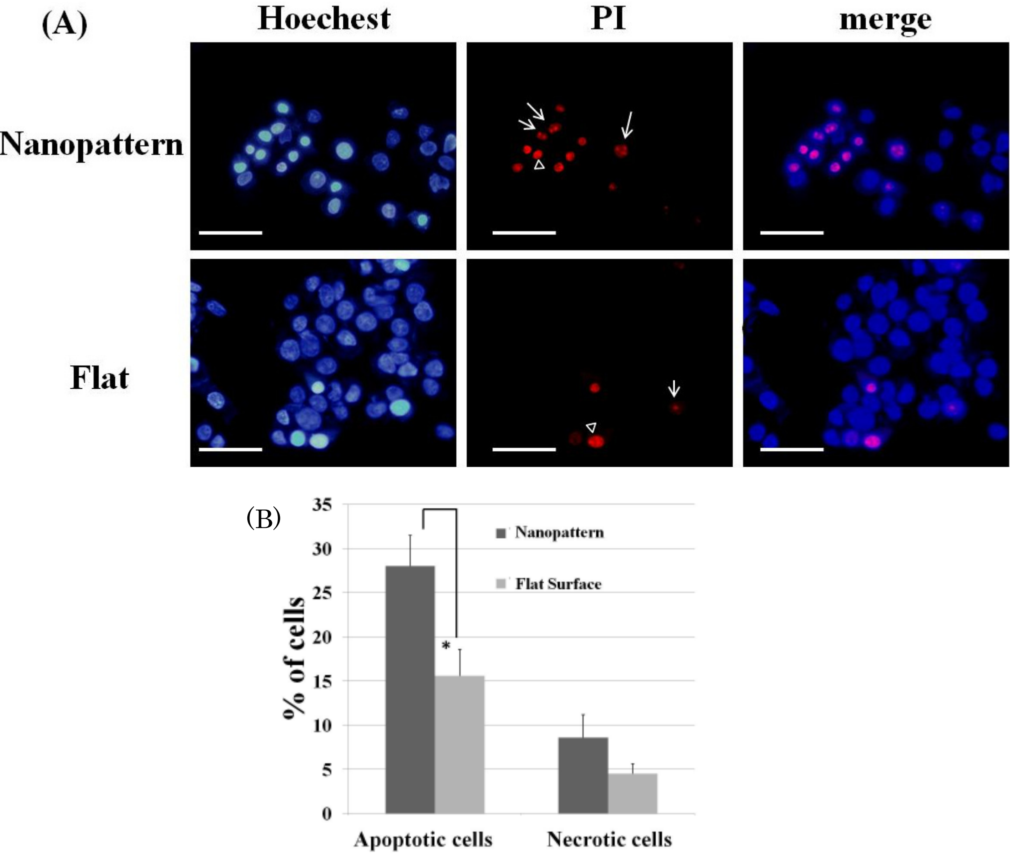


Figure 4-4: (A) Fluorescent staining of HepG2 cellular nuclei cultured on nanopattern (upper panel) and a flat surface (lower panel). Arrows show phenotype I (apoptotic cells), and arrowheads show phenotype II (necrotic cells) (Scale bar = 50 µm) (B) Alteration in number of cells cultured on various substrates showing different phenotypes of chromatin condensation induced by the presence of cisplatin. The percentage of apoptic phenotype induced by cisplatin was significantly higher in cultures on the nanopattern substrate. *: P <0.001.

3.3.1. Alterations in chromatin condensation

A significant increase (2 fold increase) in cisplatin-induced chromatin condensation was observed after culturing HepG2 cells on the nanopattern compared to the flat substrate. Figure 4-4 shows fluorescent staining of HepG2 nuclei with Hoechst (blue) and PI (red) and the percentages of these cells cultured on various substrates. The presence of cisplatin

induces different chromatin condensations in HepG2 cells compared to controls (no drug) however, the nanopattern stimulates significantly these inductions, especially to a specific nuclear phenotype (type I) or apoptotic cells relative to the flat surface ($P < 0.001$) (Figure 4-4B).

3.3.2. Morphology, cellular alignment and orientation

HepG2 cultured on the nanopattern substrate without cisplatin displayed a fundamentally different morphology than cells cultured on the flat surface. Cellular alignment in the longitudinal direction parallel to the nanogratings was maintained with an alteration in cellular spreading. Furthermore, rearrangement of actin filaments in bundle-like structures parallel to the nanograting axes was observed. However, cells cultured on flat surfaces did not shown such alignment (Figure 4-5:a-b). Moreover, filopodia fixation was observed by SEM which creates anchoring points in the planer interspace area between such nanostructures. (Figure 4-5a, inset). In the presence of a low cisplatin concentration (0.25 mM), further actin polymerization and remodeling was observed. As cells cultured over the nanopattern, the maintenance of topography-induced parallel cellular alignment was observed (Figure 4-5c) with subsequent formation of orderly arranged filopodia. Furthermore, the appearance of small blebs due to apoptosis on the cellular interface was observed. Meanwhile, for the cells cultured over the flat surface, cisplatin induced the reorganization of actin filaments with remodeling especially in the cellular periphery (Figure 4-5d).

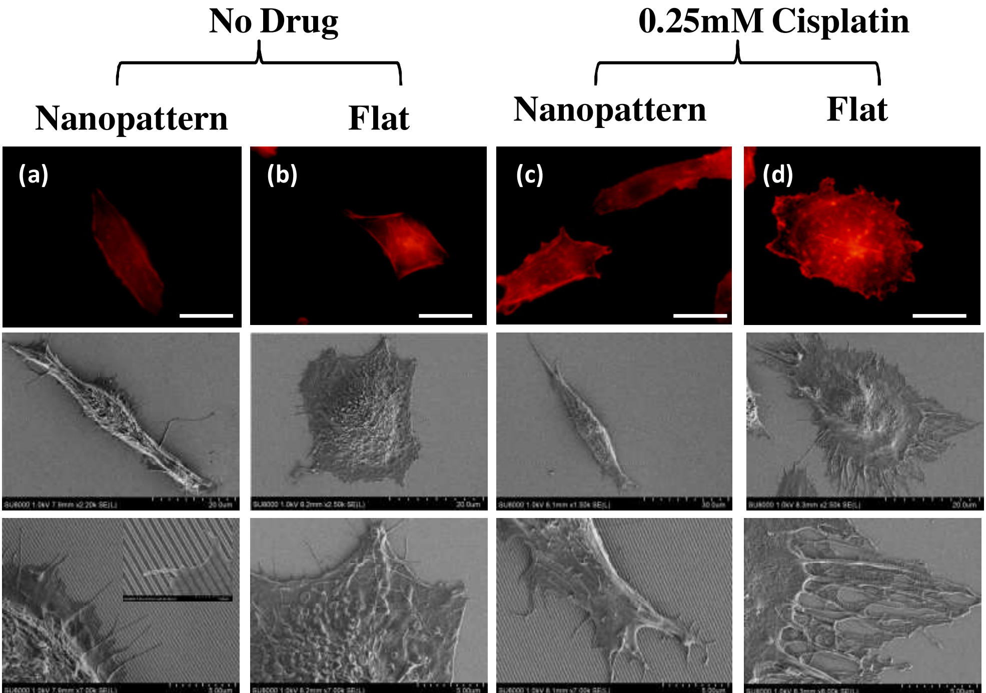


Figure 4-5: Actin filament rearrangement and morphological alteration with subsequent cellular alignment parallel to nanogratings upon culturing of cells on the nanopattern (a) or a flat surface (b) without any drug then nanopattern (c) or a flat surface (d) with 0.25 mM Cisplatin (Scale bar = 20 µm).

In a higher cisplatin concentration such as 0.5 mM, the formation of irregular cytoplasmic ledges was observed with an increase in the number of blebbing cells (Figure 4-6a), while the cellular alignment and orientation parallel to the nanopattern were maintained with their exploring filopodia (Figure 4-6a). However, at this concentration (0.5 mM), the flat substrate maintained actin filament reorganization with immoderate cytoplasmic tree-like extensions (Figure 4-6b) and morphological cellular anomalies. Furthermore, upon the use of 0.75mM cisplatin, the cells cultured over the nanopattern started to lose their nanograting recognition capabilities with subsequent loss of cellular alignment and parallel orientation rearrangements (Figure 4-6c). However, at this

concentration cells cultured on flat surfaces maintained their eccentric cytoplasmic contoured irregularities with the formation of large blebs (Figure 4-6d).

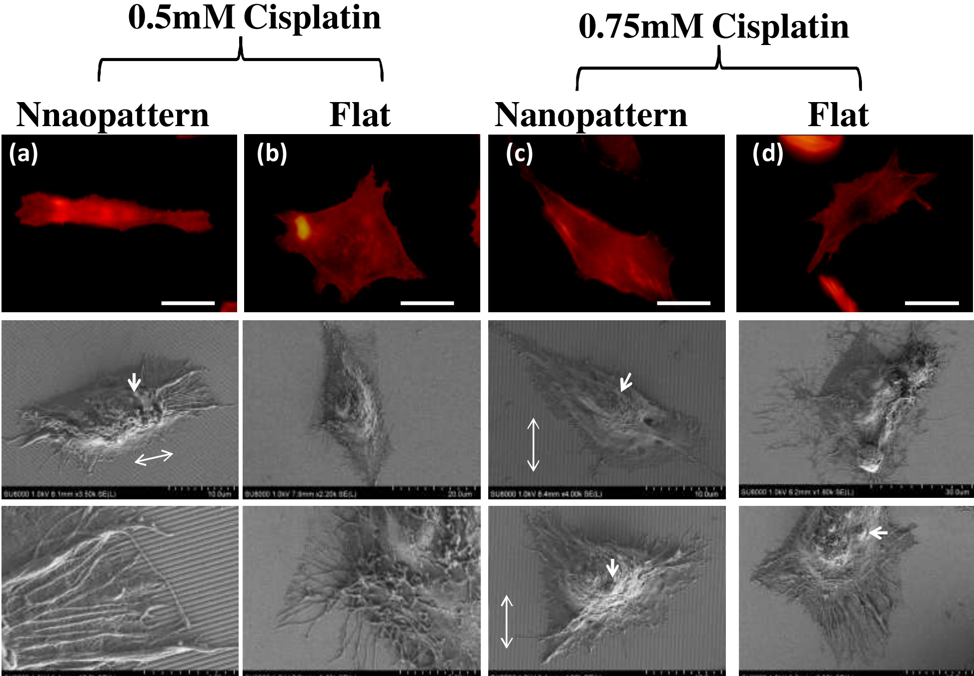


Figure 4-6: Morphological alteration induced by culturing HepG2 cells over nanopattern (a) and flat (b) substrate with 0.5 mm cisplatin concentration and nanopattern (c) and flat (d) substrate with 0.75 mm cisplatin (Scale bar = 20 μm). The formations of excessive cytoplasmic extensions and irregularities were observed with actin rearrangements followed by subsequent cellular misalignment to the nanogratings upon culturing cells on the nanopattern at higher concentrations of cisplatin (0.75 mM). Double arrows represent the directions of the nanopattern, and arrows show blebs formed as a result of apoptosis.

4. Experimental & methodology

4.1. Fabrication of nanopattern substrates using electron beam lithography and atomic layer deposition

Nanopattern substrates (240nm total dimension with gratings shape) were fabricated as previously described in chapter 3.

4.2 Hepatocyte cell culture

HepG2 were cultured in Dulbecco's MEM (Nacalai Tesque, Kyoto, Japan) with 10% heat inactivated FBS and supplemented with 100 U penicillin/100 μg streptomycin (Nacalai Tesque, Kyoto, Japan) per each ml medium. All cells were maintained at 37 °C in a 100% humidified atmosphere under 5% CO_2. At 70-80% confluency, cells were trypsinized and seeded over the nanopattern surfaces for 8 hrs. Freshly prepared cisplatin (Sigma-Aldrich, St. Louis, OM, USA) solution in DMSO was prepared for each experiment, and 0.25 mM, 0.5 mM, and 0.75 mM dilutions in DMEM were then prepared (DMSO concentration in final solution was less than 0.1%). Then cells over pattern and flat substrates were exposed to the solutions and incubated for another 12 hrs.

4.3. Live/dead viability assay

HepG2 cells cultured on nanopattern substrates and incubated with different concentrations of cisplatin were washed with PBS 3 times, then a live/dead viability kit (Invitrogen, Eugene, OR, USA) was used. The kit uses the non-fluorescent dye calcein AM (1 μM), which is enzymatically hydrolyzed into a green fluorescent dye in living cells (excitation 495, emission 515), while the impermeable fluorescent dye ethidium-D-1 passes into to dead cells and binds to nucleic acids to produce red fluorescence (excitation 595, emission 635). Observation was performed using an upright fluorescent microscope (Olympus BX51, Tokyo, Japan) equipped with an Olympus DP70 digital camera, and DP Controller Ver. 3.1.1 was used to process the images. The percentage of dead cells in a constant area (1.44 mm^2) was calculated as the number of dead cells/total number of cells × 100, each experiment was repeated at least 5 times, and statistical analysis was performed.

4.4. Immunostaining and fluorescence detection

HepG2 cells cultured on nanopattern substrates were fixed for 15 min in 4% paraformaldehyde (PFA) in PBS at 4 °C, and quenching of excess aldehyde was done using 0.1 M glycine for 5 min. Then, cells were permeabilized in 1% triton X-100 in PBS for 5 min. After that, primary antibodies were added: rabbit polyclonal anti-CTR-1 was diluted 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and added for 1 hr, goat polyclonal anti-rabbit IgG-H&L-DyLight® 488 diluted 1:500 (Abcam, UK) was added for additional 1 hr, then samples were observed using fluorescent microscopy. Cytoskeletal F-actin was visualized by treating the cells with phalloidin-TRIC (Sigma-Aldrich, St. Louis, MO, USA) at 5 μg/ml for 15 min.

4.5 Examination of chromatin condensation

HepG2 cells cultured on nanopattern substrates and incubated with cisplatin were washed with PBS 3 times, and nuclear staining was done using Hoechst 33342 (Dijindo Molecular Technology Inc., Japan) at 5 μg/ml and propidium iodide (Dijindo Molecular Technology Inc., Japan) at 1 μg/ml) for 5 min, followed by washing with PBS (2 times). Observation was performed using an upright fluorescence microscope (Olympus BX51) equipped with an Olympus DP70 digital camera. DP Controller Ver. 2.1.1 (Olympus) was used to process the images.

4.6 Scanning electron microscopy investigation

Investigations of cell rearrangements and alignments were done by SEM using a Hitachi S-4800. Prior to SEM, the samples were prepared as follows. At the end of the culture period, the cells on the substrates were fixed with 2.5 vol.% glutardialdehyde (Wako, Japan) in PBS for 2 h. After that, the cells were dehydrated through a series of ethanol concentrations (10, 40, 60, 80, 100%) for 5 min, respectively. Final desiccation was done

using freeze drying (Hitachi Es 2030, Tokyo, Japan). Finally, the samples were placed on SEM specimen holders and observed under a field-emission SEM operated at an acceleration voltage in the range 1-5 kV.

5. Discussion

ECM physical cues (i.e. topography) are responsible for the activation of intricate networks of signaling pathways through transmembrane receptor integrins. These receptors transfer extracellular mechanical signals into cellular responses by the activation of several signaling pathways and proteins such as MAPKs (mitogen activated kinases) [23]. ERK (extracellular signaling receptor kinase) is one subgroup of MAPK proteins stimulated by interactions with the extracellular major cues, with subsequent activations of cellular cascades and the control of diversified cellular behaviors [24]. Several studies have reported an important role for MAPK activations in cisplatin toxicity, especially ERK [25-29]. However, the definitive function of ERK activation and its relationship with cisplatin-induced apoptosis are not fully understood [30, 31]. ERK activation following cisplatin exposure has resulted in an increase [27-29] and occasionally a decrease [25, 26] in the cytotoxicity induced by cisplatin. These contradictory results have been explained by the sophisticated relationship between ERK activation and subsequent cascades induced by cisplatin, which mainly depend on the individual cell type and level of stress [31]. Accordingly, the coincidence between some of ERK signaling cascades activated by cisplatin and nanotopography simultaneously could be the one of suggested reasons for such observed alteration in cellular response. However, deep studies and explorations of such relationship are prerequisites before we can answer this question. So far in this study, dimensionally and geometrically well-defined nanostructures with 240 nm lateral dimension were fabricated using electron beam lithography and atomic layer deposition. Then the

examinations of their influence on the cellular behaviors of hepatic cell line (HepG2) after cisplatin exposure were carried on short time course.

Since a higher cytotoxic response was observed on the nanopattern relative to the flat surface, these suggest a role for nanotopography and the simulation of ECM intrinsic elements in the altered cytotoxic response to cisplatin. While we do not know exactly what the underlying mechanism of such variation in cytotoxicity was, but we can consider four factors: 1) cisplatin uptake, 2) alteration in intracellular signaling, 3) intracellular efflux of cisplatin, 4) inactivation of cisplatin. Any change in these factors induced by nanotopography could be responsible for the altered cytotoxic response. Manipulation of cisplatin uptake is a tempting explanation for our observations, since cisplatin uptake is regulated by a variety of mechanisms [32, 33] that include passive diffusion, endocytosis, and CTR-1 receptors. Micro and nanotopography have been previously reported to modify the cellular internalization of small molecular weight chemicals and DNA by endocytosis [34]. The existence of micro/nanosized features induces a wide array of cellular responses, especially morphological changes, with the subsequent formation of densely packed actin filaments [35-39]. The resulting rearrangement of these filaments affects cellular contractility and upregulates the Rho-GTPase pathway, which enhances different mechanism of enodcytosis. Nevertheless, this topography-induced endocytosis is dependent not only on the superficial characteristics of topography but also on the cell type [34]. Consequently, such topography-induced cellular internalization is a strong candidate for explaining the observed increase in the cytotoxic response; however, there may be multiple mechanisms since nanotopography induced wide array of cellular cascades. What we can conclude is that ECM physical cues such as topography play a role in the alteration of cytotoxic responses to cisplatin in these hepatic cells.

Copper transporter-1 (CTR-1) is a channel-like transporter membrane protein with 3 transmembrane domains [40]. It has a key role in the initial influx and active transport of cisplatin and other platinum drugs [41]. Alterations in cisplatin uptake have been associated with the presence or absence of these receptors [42], and the deletion of CTR-1 from yeast and fibroblasts is responsible for an 80% reduction in cisplatin uptake [43]. Furthermore, CTR-1-overexpressing cells have significantly increased intracellular accumulation of cisplatin [44]. Consequently, the level of CTR-1 is significantly modulated by the mimicing of natural ECM cues induced by the 240 nm nanopattern, since better cytocompatibility and functionality of HepG2 are induced by this nanopattern [21]. We cannot conclude that the observed increase in CTR-1 expression is the only reason for the topography-induced modified cytotoxic response. However, we suggest that it plays a role in addition to other previously mention mechanisms. As the underlying mechanism for the alteration is not fully clear, we will further explore these results in future studies. As a deeper understanding of the relationship between the topographical features of ECM and cellular behavior could help clinicians modulate cytotoxic drug responses. Furthermore, it could have a promising role in the further recognition of intrinsic cytotoxic drug responses and the unique structural determinants of ECM for such a response.

Cellular exposure to cisplatin is associated with apoptosis and the formation of nuclear structural rearrangements and chromatin condensation [45]. Such morphological variations could be observed and differentiated using fluorescence staining of nuclei with Hoechst and propidium iodide (PI), which allowed differentiation of apoptosis and necrosis. Apoptotic cells are double-stained with Hoechst with condensed nuclei with PI-stained spots, while necrotic cells have large nuclei intensely stained with Hoechst and PI [46]. This phenotype represents cells with explicit signs of apoptosis such as a decrease in cellular size and

chromatin condensation with subsequent localization in nuclear ledges (Figure 4-4a). However, the percentage of the other nuclear phenotype (necrosis) stimulated by cisplatin did not change upon culturing HepG2 cells over the nanopattern compared to the flat substrate (Figure 4-4b). These results emphasize that cisplatin induced apoptosis was increased on nanopattern compared to flat surface, in another words, the nanopattern encounters to mimic the ECM physical cues following cisplatin exposure aggravated the cisplatin cytotoxicity, indeed, the underlying mechanism for such observation needs further study at molecular level and different pathways which could be influenced by such cues.

Cell morphology in general and the rearrangement of actin filaments in particular are major determinants of cellular behavior. Actin is a major cytoskeletal protein that polymerizes within cells to form thin, flexible fibers (filaments) approximately 7 nm in diameter and up to several micrometers in length. These filaments are organized into highly ordered structures, forming bundles or three-dimensional networks. The assembly and disassembly of actin filaments provide mechanical support, determine cell shape, and finally allow for movement of the cell surface, migration, and endocytosis [47]. Furthermore, the continuous remodeling and polymerization of actin filaments play a key role in oncogenic signaling pathways as well as ultrastructural variations associated with malignant cells [48].

These results indicated that the morphological changes that are characteristic of cisplatin exposure were modified due to the presence of physical cues that mimic the ECM. These concentration-dependent alterations include changes in surface characteristics, cellular shape, and alignment. Consequently, these observations underline the role of the cellular microenvironment in drug sensitivity, and are critical for future studies into the mechanisms and structural determinants of drug resistance of cancer cells.

6. Conclusions

In summary, we examined the toxicity of cisplatin for hepatic cells in presence of manipulated substrates that mimic ECM cues. The topography manipulation as a major determinant of cytotoxic responses of a drug *in-vitro* was observed. Since a modulation of topography greatly increased the cytotoxicity induced by cisplatin. A higher cytotoxic response was observed when cells were cultured over the nanopattern relative to the flat surface. Furthermore, alterations in substratum topography in the presence of cisplatin induced transformations in chromatin condensation, cellular shape, orientation, and cell alignment relative to a flat surface. Thus, we conclude the important role of ECM elemental cue 'topography' in the manipulation of hepatocellular responses to a cytotoxic agent. A deep understanding of this role for a wide array of cells could be further exploited to produce distinguished biomimetic surfaces. Such surface could be utilized for the development of more accurate and sensitive drug-toxicity analysis.

7. References

[1] Pamula E, De Cupere V, Dufrene YF, Rouxhet PG, Nanoscale organization of adsorbed collagen: influence of substrate hydrophobicity and adsorption time, J Colloid Interface Sci., 2004; 271: 80-91.

[2] Gasiorowski JZ, Murphy CJ, Nealey PF, Biophysical Cues and Cell Behavior: The Big Impact of Little Things, Ann Rev Biomed Eng., 2013; 15: 155-176.

[3] Hubmacher D, Apte SS, The biology of the extracellular matrix: novel insights, Curr Opin Rheumatol., 2013; 25: 65-70.

[4] Lukashev ME, Werb Z, ECM signalling: orchestrating cell behavior and misbehavior, Trends Cell Boi., 1998; 8: 437–441.

[5] Watt FM, Huck WTS, Role of the extracellular matrix in regulating stem cell fate, Nat Rev Mol Cell Biol., 2013; 14: 467–473.

[6] Meng Q, Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity, Expert Opin Drug Metab Toxicol., 2010; 6: 733-746.

[7] Bruinink A, Bitar M, Pleskova M, Wick P, Krug HF, Maniura-Weber K, Addition of nanoscaled bioinspired surface features: A revolution for bone-related implants and scaffolds?, J Biomed Mat Res Part A., 2014; 102: 275-294.

[8] Carloni V, Luong TV, Rombouts K, Hepatic stellate cells and extracellular matrix in hepatocellular carcinoma: more complicated than ever, liver internationals, DOI:10.1111/liv.12465

[9] Nguyen-Ngoc K-V, Cheung K J, Brenot A, Shamir Ryan ER, Gray S, Hines WC, Yaswen P, Werb Z, Ewald AJ, ECM microenvironment regulates collective migration and local dissemination in normal and malignant mammary epithelium, Proc Nat Acad Sci., 2102; 109: E2595–E2604.

[10] Yang C, Zeisberg M, Lively J C, Nyberg P, Afdhal N, Kalluri R, Integrin α1β1 and α2β1 Are the Key Regulators of Hepatocarcinoma Cell Invasion Across the Fibrotic Matrix Microenvironment, Cancer Res,, 2003; 63: 8312-8317.

[11] Goetz JG, Minguet S, Navarro-Lérida I, Lazcano JJ, et al., Biomechanical Remodeling of the Microenvironment by Stromal Caveolin-1 Favors Tumor Invasion and Metastasis, Cell, 2011; 146: 148-163.

[12] Paszek MJ, Zahir N, Johnson KR, Lakins JN, Rozenberg GI, et al., Tensional homeostasis and the malignant phenotype Cancer, Cell, 2005; 8: 241–254.

[13] Ingber DE, Madri JA, Jamieson JD, Neoplastic disorganization of pancreatic epithelial cell-cell relations: Role of basement membrane, Am J Pathol., 1985; 121: 248–260.

[14] Wang F, Hansen RK, Radisky D, Yoneda T, Barcellos-Hoff MH, Petersen OW, Turley EA, Bissell MJ, Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts, J Natl Cancer Inst., 2002; 94: 1494–503

[15] Sethi T, Rintoul RC, Moore SM, MacKinnon AC, Salter D, Choo C, et al., Extracellular matrix proteins protect small cell lung cancer cells against apoptosis: a mechanism for small cell lung cancer growth and drug resistance in vivo, Nat Med., 1995; 5: 662-668.

[16] Conti JA, Kendall TJ, Bateman A, Armstrong TA, Papa-Adams A, Xu Q, et al., The desmoplastic reaction surrounding hepatic colorectal adenocarcinoma metastases aids tumor growth and survival via alpha(v) integrin ligation, Clin Cancer Res., 2008; 14: 6405-6413.

[17] Schrader J, Gordon-Walker TT, Aucott RL, Deemter MV, Quaas A, Walsh S, Benten D, Forbes SJ, Wells RG, Iredale JP, Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells, Hepatology, 2011; 53: 1192–1220.

[18] Levental KR, Yu H, Kass L, Lakins JN, Egeblad M, Erler JT, et al., Matrix crosslinking forces tumor progression by enhancing integrin signaling, Cell, 2009; 139: 891-906.

[19] Bokhari M., Carnachan RJ, Cameron NR, Przyborski SA, Culture of HepG2 liver cells on three dimensional polystyrene scaffolds enhances cell structure and function during toxicological challenge, J Anat., 2007; 211:567–576

[20] Kievit FM, Florczyk SJ, Leung MC, Veiseh O, Park JO, Disis ML, Zhang M, Chitosan–alginate 3D scaffolds as a mimic of the glioma tumor microenvironment, Biomaterials, 2010; 31: 5903–5910.

[21] Abdellatef S A, Ohi A, Nabatame T, Taniguchi A, Induction of hepatocyte functional protein expression by submicron/nano-patterning substrates to mimic in vivo structures, Biomater Sci., 2014; 2: 330–338.

[22] El-Sayyad HI, Ismail MF, Shalaby Fm, Abou-El-Magd RF, Gaur RL, Fernando A, Raj MHG, Ouhtit A, Histopathological effects of cisplatin, doxorubicin and 5-flurouracil (5-FU) on the liver of male albino rats. Int J Biol Sci., 2009; 5: 466-473.

[22] Biggs MJP, Richards RG, Gadegaard N, Wilkinson CDW, Oreffo ROC, Dalby MJ, The use of nanoscale topography to modulate the dynamics of adhesion formation in primary osteoblasts and ERK/MAPK signalling in STRO-1+ enriched skeletal stem cells, Biomaterials, 2009; 30: 5094–5103.

[23] Biggs MJP, Richards RG, Dalby MJ, Nanotopographical modification: a regulator of cellular function through focal adhesions, Nanomed: Nanotechnol Bio Med., 2010; 6: 619–633.

[24] Wei SQ, Sui LH, Zheng JH, Zhang GM, Kao YL, Role of ERK1/2 kinase in cisplatin-induced apoptosis in human ovarian carcinoma cells, Chin Med Sci J., 2004; 19: 125–129.

[25] Hayakawa J, Ohmichi M, Kurachi H, Ikegami H, Kimura A, Matsuoka T, Jikihara H, Mercola D, Murata Y, Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line, J Biol Chem., 1999; 274: 31648–31654.

[26] Woessmann W, Chen X, Borkhardt A, Ras-mediated activation of ERK by cisplatin induces cell death independently of p53 in osteosarcoma and neuroblastoma cell lines, Cancer Chemother Pharmacol., 2002; 50: 397–404.

[27] Wang X, Martindale JL, Holbrook NJ, Requirement for ERK activation in cisplatin-induced apoptosis, J Biol Chem., 2000; 275: 39435–39443.

[28] Nowak G, Protein kinase C-alpha and ERK1/2 mediate mitochondrial dysfunction, decreases in active Na+ transport, and cisplatin-induced apoptosis in renal cells, J Biol Chem., 2002; 277: 43377–43388.

[29] Brozovic A, Osmak M, Activation of mitogen-activated protein kinases by cisplatin and their role in cisplatin-resistance, Cancer Lett., 2007; 251: 1–16.

[30] Wang D, Lippard SJ, Cellular Processing Of Platinum Anticancer Drugs, Nat Rev Drug Dis., 2005; 4: 307-320.

[31] Arnesano F, Natile G, Mechanistic insight into cellular uptake and processing of cisplatin 30 years after its approval by FDA coordination, chem rev., 2009; 253: 2070-2081.

[32] Safaei R, Katano K, Samimi G, Holzer AK, Naerdemann W, Howell SB, Contribution of endocytic pathways to the uptake of cisplatin in sensitive and resistant ovarian cancer cells. AACR Meeting Abstracts, 2004: 120-b.

[33] Teo BK, Goh SH, Kustandi TS, Loh WW, Low HY, Yim EK, The effect of micro and nanotopography on endocytosis in drug and gene delivery systems, Biomaterials, 2011; 32: 9866-75.

[34] Kim J, Kim HN, Lim K-T, Kim Y, Seonwoo H, Park SH, Lim HJ, Kim D-H, Suh K-Y, Choung P-H, Choung Y-H, Chung JH, Designing nanotopographical density of extracellular matrix for controlled morphology and function of human mesenchymal stem cells, Scientific reports , 2013; 3: 1-11.

[35] Teixeira AI, Nealey PF, Murphy CJ, Responses of human keratocytes to micro- and nanostructured substrates, J Biomed Mater Res Part A., 2004; 71A: 369-376.

[36] Wang P-Y, Yu J, Lin J-H, Tsai W-B, Modulation of alignment, elongation and contraction of cardiomyocytes through a combination of nanotopography and rigidity of substrates, Acta Biomater., 2011; 7: 3285–3293.

[37] Lamers E, Horssen RV, Riet JT, Van Delft FCMJM, Luttge R, Walboomers XF, Jansen J A, The Influence of Nanoscale Topographical Cues on Initial Osteoblast Morphology and Migration Euro, Cells & Mater., 2010; 20: 329-343.

[38] Yim EKF, Darling EM, Kulangara K, Guilak F, Leong KW, Nanotopography-induced changes in focal adhesions, cytoskeletal organization, and mechanical properties of human mesenchymal stem cells, Biomaterials, 2010; 31: 1299–1306.

[39] Nose Y, Rees EM, Thiele DJ, Structure of the Ctr1 copper transporter reveals novel architecture, Trends in Biochem Sci., 2006; 31: 604–607.

[40] Safaei R, Howell SB, Copper transporters regulate the cellular pharmacology and sensitivity to Pt drugs, Crit Rev Oncol Hematol., 2005; 53: 13-23.

[41] Ciarimboli G, Membrane Transporters as Mediators of Cisplatin Effects and Side Effects, Scientifica, 2012: 1-18.

[42] Ishida S, Lee J, Thiele DJ, Herskowitz I, Uptake of the anticancer drug cisplatin mediated by the copper transporter Ctr1 in yeast and mammals, Proc Nati Acad Sci., 2002; 99: 14298–14302.

[43] Holzer AK, Samimi G, Katano K et al., the copper influx transporter human copper transport protein 1 regulates the uptake of cisplatin in human ovarian carcinoma cells, Mol Pharm.,2004; 66: 817–823.

[44] Ramos JM, Arrebola F, Fernández-Cervilla FJ, Crespo V, Fernández-Segura E, Electron Probe X-Ray Microanalysis of Cisplatin Induced Cell Death in Rate Phenochromocytoma PC12, Cells Histol Histopathol., 2012; 26: 333-342.

[45] Anestål K, Arnér ESJ, Enzyme Containing Selenocysteine Reductase 1 but Not by the Fully Active Selenium-compromised Thioredoxin Rapid Induction of Cell Death, J Biol Chem., 2003; 278: 15966-15972.

[46] Cooper GM, The Cell: A Molecular Approach. 2nd edition. Sunderland (MA): Sinauer Associates; 2000. Structure and Organization of Actin Filaments. Available from: http://www.ncbi.nlm.nih.gov/books/NBK9908/

[47] Gonçalves EM, Ventura CA, Yano T, Rodrigues Macedo ML, Genari SC, Morphological and growth alterations in Vero cells transformed by cisplatin, Cell Biol Int., 2006; 30: 485-94.

Chapter 5

Conclusion Remarks and future aspects

1. Conclusions

In this work I tried to overcome some of challenges associated with the utilization of hepatic cells for *in-vitro* drug analysis applications. Since the decreased in functionalities is one of the major problems that hinders the validity and accuracy of such cells for pharmaceutical industries and drug analysis strategies. So I tried to determine the influence of mimicking various ECM cues by physically and chemically manipulate the surface of substrates and the hepatocellular responses and functionalities. Since mimicking of the native cellular environment or ECM is a fundamental process for the development of biomaterials with improved cell integration properties and functionalities. As such, many have focused their research about the development of various techniques and methodologies for mimicking such ECM cues (as stiffness, topography, ECM compositions) to try to illustrate the essential factors that simulate the ECM cues and its role in the promotion of cellular behaviors. Understanding such factors on which specific superficial characteristics of material can promote or decrease the cellular performance is important for the development of cell instructive biomaterials with specific cellular integration properties especially to be utilized for the improvement of current culture substrates. Furthermore, cellular identities likewise material characteristics play the key role in the determination of such cell-material interactions.

Consequently, some tangible superficial characteristics of biomaterials that can be involved in production of cell culture substrates for hepatic cells with more valid *in-vitro* drug analysis were identified at the end of this research. Even though several factors and cues have not been analyzed yet that could be utilized in the fabrications of cell instructive

biomaterials for hepatocellular applications (figure 5-1) some of these factors will be illustrated in future aspects.

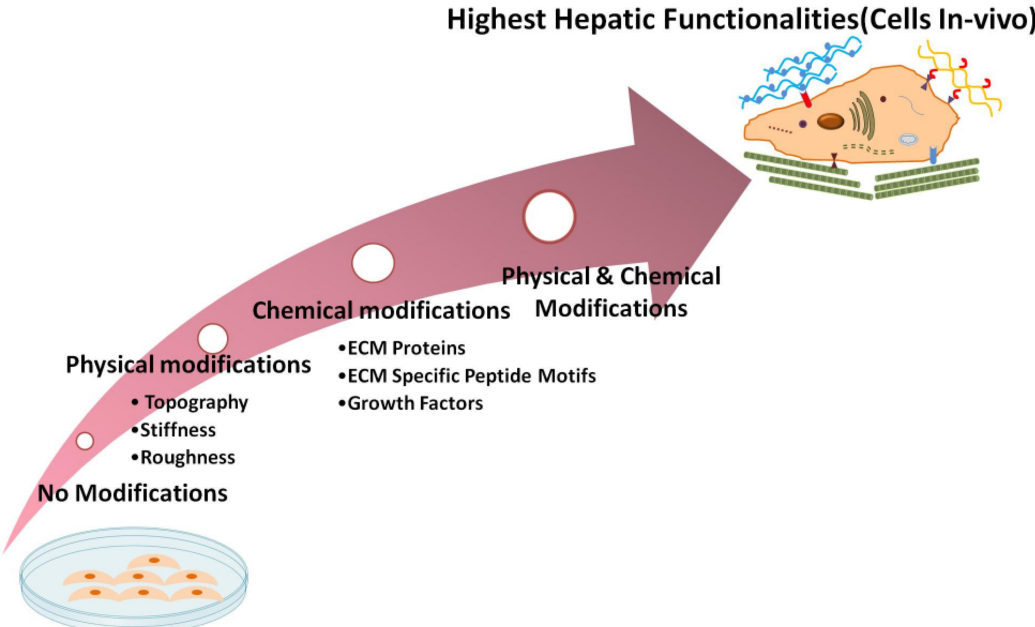


Figure 1-5: The steps to increase the hepatocellular functionalities by mimicking some of ECM elemental cues in-vivo as topography or peptide motifs.

The **first Chapter** contains an introduction regarding the importance and functionalities of liver cells inside the body and in pharmaceutical industries after that the differences between hepatocellular responses *in-vitro* and *in-vivo* are illustrated. A brief definition of ECM and its functionalities as a possible reason for such discrepancies are mentioned. The explanations of various ECM cues with a description of the available methodologies to mimic liver ECM were written. The reasons for the utilization of synthetic biomaterial for *in-vitro* tissue culture substrates, the role of material topography to control the hepatocellular responses, the role of RGD as a chemical modulator for hepatic behaviors were explained. Finally, the motivations and purpose of such work are illustrated, followed by an explanation

of my hypothesis, work strategies, with an illustration of the relationship between our fabricated nanopattern's characteristics and native ECM cues, after that the reasons for the use of TiO_2 film surface coating for our nanopattern and flat substrates. Detailed work strategies and results are described in chapter 2-4.

The **second chapter** describes the relationship between the manipulation of geometrical and dimensional characteristics of nanopattern and the control of hepatic cell line behaviors. Since such used nanopatterns were fabricated by electron beam lithography and atomic layer deposition. The morphological and functional responses in the hepatic cell line induced by such diversified nanofeatures were characterized using fluorescent immunostaining techniques. The expressions of functional proteins such as albumin, transferrin and cytochrome P450 were tested as functional markers. The interactions of such hepatic cell line with nanopatterns, which have heterotropic dimensions, may regulate cellular function since the increase of the interspace between nanogratings was associated with a decrease in the hepatocellular functionalities. Furthermore, the geometry likewise dimension of nanofeatures could be effective tool to control cellular behaviors, since the alteration of hierarchically extended collagen like structures from a continuous linear shape to intermittent rectangles lowered the cellular functionality of HepG2 cells. Nanopattern with grating shape and 240 nm showed more than 6 folds increase in the production of albumin compared to flat surfaces. So we can conclude that biofunctionality and cytocompatibility of hepatic cell line can be increased by the physical variation in substrates as topography. Edge to edge spacings and feature models, are important determinants for the increase of hepatocellular functionalities. This substratum topography with significantly increased hepatic functionalities can be used for the development of cell culture substrates that can be utilized in more accurate *in-vitro* drug analysis and testings Not only functional protein secretions

were affected by shape of topography but also the manipulations of cellular orientation, cell alignment and native extracellular matrix (ECM) assembly were induced by the geometry of such nanotopographies. Topography manipulations to mimic ECM cues in substrates can be utilized not only to increase functional protein expression (Figure 5-1). Consequently, physical characteristics of nanofeatures as size and geometry are crucial regulatory determinant of hepatocellular responses and functionalities since cells were able to transduce the alteration of superficial characteristics with subsequent change in the functional and orientational aspects.

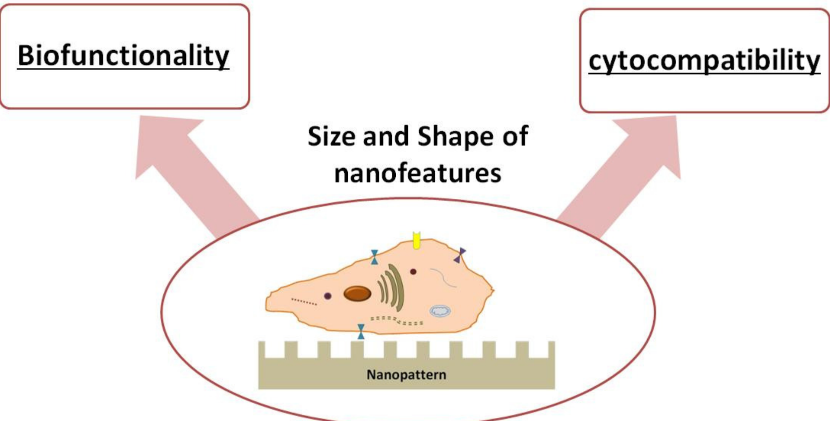


Figure 5-1: Depicts the influence of physical variations as size and shape of nanostructures for the modulation of cellular functionality and cytocompatibility.

The **third Chapter** describes the integration of chemical and physical ECM cues in a single substrate and role of such integration on the hepatocellular responses. This was done by the incorporation of organic moieties with specified cellular functionality, such as RGD motifs, into the deposited TiO_2 inorganic film using an enzyme catalyzed oxidation reaction. The role of simultaneously combining such diversified cues on the morphological and functional response of HepG2 cells was investigated using fluorescent immunostaining techniques. A significant enhancement in the expression of liver-specific markers was

observed on RGD-coated surfaces compared to uncoated substrates regardless of topography. This emphasizes the major role of chemical cues in enhancing the hepatic functionality compared to substratum topography alone. Furthermore, a synergism is observed due to the combination of such cues in a single substrate especially for the expression of liver-specific markers such as albumin, since nearly the integration of these has resulted nearly 1.6 fold increase in expression of albumin compared to RGD-Flat surfaces. Thus, the presence of nanofeatures with well-defined dimensions and chemical composition that imitate ECM fibrillar structures and containing a specific motif (e.g. RGD) could be utilized to increase multiple cellular functions, rather than simply using nanotopography or chemical cues alone. Therefore, the greater the ECM intrinsic elements are mimicked, the better the substrate's cytocompatibility and the functionality of HepG2.

The **fourth chapter** describes the utilization of nanostructures that mimic ECM physical cues as a culture substrate for *in-vitro* analysis of drug cytotoxicity. The resulted hepatocellular cytotoxic responses and morphological changes due to the presence of such nanofeatures after exposure to a cytotoxic agent "cisplatin" were investigated using fluorescent immunostaining techniques. A significant increase in the percentage of dead cells (nearly 2 fold increase) upon the change of topography after cisplatin exposure relative to flat surface was observed. This emphasizes the role of simulation of ECM intrinsic elements (as physical cues) in the alteration of cytotoxic response to cisplatin. Moreover, such alteration in substratum topography in presence of cisplatin induce a transformation in nuclear chromatin condensations, morphological variabilities and its directional cellular alignments compared to flat surface. The formation of irregular cytoplasmic ledges was observed with an increase in the number of blebbing cells for those cultured on nanopattern

in cisplatin-concentration dependant manner, while the cellular alignments were concomitantly disrupted. I can conclude that mimic the biophysical characteristics of ECM are extremely important determinants of cellular response in a cytotoxic platform for development of more sensitive *in-vitro* drug analyses.

Finally, we can conclude that **more improved hepatocellular functionalities and increased cytotoxic responses which are beneficial for accurate *in-vitro* drug analyses can be developed by the manipulation of surface characteristics of the culture substrates.** These alterations could include the physical and chemical manipulations of superficial characteristics in order to promote the hepatocellular behaviors and functionalities. Such manipulations should closely mimic the natural characteristics of ECM elemental cues or cellular native environments for the control of cellular response. Since the role of biomaterial manipulation; superficial texturing, chemical composition and topographical feature to control hepatic cellular behaviors and functionality were emphasized.

2. Future aspects

In this work, I was able to recognize some of tangible cues to alter hepatocellular behaviors in general and functionalities in special. Such cues could be used for the development of novel biomaterials culture models, since some of essential structural determinants of ECM cues required for the metallic based biomaterial in liver cells' applications were identified that could be further exploited for drug development applications. While the underlying mechanisms for this enhanced functionality and altered response have yet to be fully revealed, and they need extensive study owing to the

sophistication of hepatic functionalities and signaling pathways on which these entangled signals are organized to some extent by such diversified physical and biochemical cues

Moreover, the role of topography manipulation and geometrical variations as an ECM structural element at the cellular level was determined however, several other geometrical variations can be examined for a further control of diversified cellular responses especially with the continuously evolved techniques to fabricate and dominate the morphologies and geometries of nanostructures.

Furthermore, in this work, we used TiO_2 films for the surface coating of our topographical nanostructures. While there are several candidates can be used instead of TiO_2 that will impart diversified surface characteristics to the nanostructures. Especially when a reported research (IA Janson, et. al. Plos one, 2014; 9: e90719) claims that nanotopography made from poly methyl(methacrylate polymer) would not induce the osteogenic differentiation of mesenchymal stem cells even though another research has reported the opposite and emphasize the role of nanotopography as an inducer for MSCs osteogenic differentiations (Watari S, et. al, Biomaterials, 2012; 33: 128-136.). IA Janson, et. al. tried to attribute such change in the cellular response to the change in the surface chemistry and other surface characteristics that may influence the identity and conformation of adsorbed proteins or the variations in size of topographies thus subsequently diversified cellular responses can be expected.

Finally, the integration of more than single ECM cues in the fabricated biomaterials goes beyond hepatic cells as it can be extended to other types of cellular models in implant technologies as well as the determination of stem cells fate.

List of publication

▪ ○ The effect of physical and chemical cues on hepatocellular function and morphology
International Journal Molecular. Science. 2014, 15, 4299-4317.
DOI: 10.3390/ijms15034299
<u>Shimaa A. Abdellatef</u>, A. Ohi, T. Nabatame, A. Taniguchi

▪ ○ Induction of hepatocyte functional protein expression by submicron/nano-pattern substrates to mimic in vivo structures,
Biomaterials Science, 2014, 2, 330–338.
DOI: 10.1039/C3BM60191A
<u>Shimaa A. Abdellatef</u>, A. Ohi, T. Nabatame, A. Taniguchi

<u>Oral Presentations</u>

1. Topography induced alteration in the cytotoxicity induced by cisplatin,
NIMS-Waseda Joint Symposium, Tsukuba, Japan (March 2014).
<u>Shimaa A. Abdellatef</u>, A. Taniguchi

2. Alteration of the hepatocellular functionality using Metallic based nanopatterns.
23rd intelligent material systems Symposium, Tokyo , Japan (January 2014)
<u>Shimaa A. Abdellatef</u>, A. Taniguchi

<u>Poster presentations</u>

1. Alteration in Hepatocytes Behaviors by the Manipulation of Chemical and Physical Cues on TiO_2 Nanopatterns.
International Conference on Nanotechnology in Medicine, London, UK (February 2014)
<u>Shimaa A. Abdellatef,</u> A. Taniguchi

2. TiO_2 Nanopattern Altering the Hepatcellular behaviours in Toxic condition,
Tsukuba Medical Engineering Cooperation Symposium, Tsukuba, Japan (January 2014).
<u>Shimaa A. Abdellatef</u>, Riho Tange, Takeshi Sato, Akiyoshi Taniguchi

3. Study of Interactions between TiO_2 Nanopatterning Surfaces and Human Liver Cell line

Annual Meeting of Japanese Biomaterial Society, Tokyo , Japan (November 2013).

Shimaa A. Abdellatef, A. Ohi, T. Nabatame, A. Taniguchi

4. TiO_2 Nanopattering That mimicks the Extracellular Matrix induced functions of human liver cells.

Advanced Material World Congress, Izmir, Cesme, Turkey (September 2013).

Shimaa A. Abdellatef, A. Ohi, T. Nabatame, A. Taniguchi

5. Study of the interactions between TiO_2 Nanopattern surfaces and human liver cells ,

Waseda/MANA NIMS International Symposium, Tsukuba, Japan (March 2013).

Shimaa A. Abdellatef, A. Ohi, T. Nabatame, A. Taniguchi: