Thermal induced coupling of cell adhesion/deadhesion and migration under HBV replication and EGCG treatment

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**THERMAL INDUCED COUPLING OF CELL
ADHESION/DEADHESION AND MIGRATION UNDER HBV
REPLICATION AND EGCG TREATMENT**

LI XI

School of Chemical and Biomedical Engineering

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- **Li X**, Feng H, Ng Soon Seng, Chen WN, Chan V. The Modulatory Behavior of Epigallocatechin-3-gallate (EGCG) in Cell Adhesion/Deadhesion and Migration of liver cancer Cells. *Submitted*
- Feng H, **Li X**, Niu DD and Chen WN. (2010) HBV replication activates RhoA GTPase: specificity of viral genotypes. *Submitted*
- **Li X**, Feng H, Chen WN, Chan V. Hepatitis B virus induced coupling of deadhesion and migration of HepG2 cells on thermo-responsive polymer. *Biomaterials*. 31(7):1894-903.
- Feng HX, **Li X**, Niu DD, Chen WN. Protein profile in HBx transfected cells: A comparative iTRAQ-coupled 2D LC-MS/MS analysis. *J Proteomics*. 2009.
- Feng HX, Zhang JH, **Li X**, Chen WN. HBX-mediated migration of HBV-replicating HepG2 cells: insights on development of hepatocellular carcinoma. *J Biomed Biotechnol*. 2009:930268.

Summary

The unique physical property of thermo-responsive polymer (TRP) has recently prompted its increasing applications in the applications of tissue engineering and regeneration medicine. On the other hand, TRP has never been exploited for their potential applications in quantitative cell screening against various external stimulations. In the first part of my thesis, TRP was applied as a model system for elucidating the effect of HBV replication on the biophysical responses of HepG2 cells transfected by wild type HBV genome. Moreover, mutant HBV genome is designed to assess the specific activity of the SH3-binding domain of HBx during HBV replication. The adhesion contact recession and geometry transformation of HepG2 cells transfected with empty vector (pcDNA3.1 cells), wild type HBV (wtHBV cells) and mutant HBV genome (mHBV cells) were probed during the thermal transformation across lower solution critical temperature of TRP. In comparison with pcDNA3.1 cells and mHBV cells, the initial rate of reduction in degree of deformation and average adhesion energy for wtHBV cells was significantly increased. Interestingly, migration speed and persistence time of cells were found to be correlated with the cell deadhesion kinetics. Immuno-fluorescence microscopy demonstrates that HBV replication reduces the actin concentration and focal adhesions at cell periphery during the initial 30 min cell deadhesion. The results strongly suggested that HBV replication triggers the dynamic responses of HepG2 cells through the cytoskeleton

remodeling and subsequent mechanochemical transduction. Overall, it is shown that TRP provides an exciting platform for quantifying biological stimulations on adherent cells.

With its widely reported cytostatic, cancer chemopreventive and tumor inhibiting properties, epigallocatechin-3-gallate (EGCG), the main polyphenolic constituent of green tea, has been widely regarded as a promising candidate of selective anticancer remedies. Till now, most studies of EGCG focused on its impact on proliferation and apoptosis. Nevertheless, the consequences of EGCG treatment on cellular activities for cells adhering on extracellular matrix remain obscure. Since cell adhesion, deadhesion, and migration are fundamental activities and essential mechanisms underlying cancer invasion and metastasis, we investigated the consequence of EGCG treatment on the biophysical behavior of cells in order to develop new approach to screen the potential therapeutic effects of EGCG. A cell line derived from human liver carcinoma, HepG2, was employed as a model system. The adhesion contact dynamics, deadhesion kinetics and geometry of HepG2 cells treated with different concentrations of EGCG during thermally triggered deadhesion were then probed by confocal-reflectance interference contrast microscopy (C-RICM) in combination with phase contrast microscopy. We observed that accelerated cell adhesion, decelerated cell deadhesion were caused by the EGCG-induced actin remodeling. Interestingly, the decreased cell motility determined from real-time cell migration assay was

consistent with the alteration of the kinetics of cell adhesion and deadhesion. With the utilization of cell biology techniques and highly integrative functional microscopy, our work brought new insights into the quantitative correlation between cell adhesion, deadhesion, migration parameters, while the potential of EGCG treatment as anticancer therapy was assessed.

After exploring the relation between adhesion/deadhesion kinetics and cell migration parameters of HepG2 cells under EGCG treatment, we demonstrated the potential role of EGCG in modulating the metastasis of liver cancer. Previously, we also accomplished the identical work on HBV genome transfected HepG2 cells. Nevertheless, the direct impact of EGCG treatment for HBV replication remains obscure. A derivative cell line of HepG2, HepG2.2.15, with enhanced HBV replication system, was employed to mimic HBV-infected cells. Thus we introduced HepG2.2.15 cell line in order to provide a tailored cell culture system with stable and sufficient HBV replication. With the utilization of real-time cell migration tracking assay, we investigated the effect of EGCG on the abnormally elevated motility of HepG2.2.15 cells on model ECM-coated TRP. The results suggested that EGCG restored the high cell motility of HepG2.2.15 towards the original level of normal HepG2 cells in a dose-dependent fashion. It was again testified that EGCG played a decisive role in the inhibition of cell migration. Through probing intricate cellular responses to external stimuli, the quantitative function of our biophysical approaches applied herein was proven to be good at

evaluating new and potential cancer therapeutics.

To simulate the early HBV infection and characterize early cellular response to HBV infection specifically associated with the proline-rich region in SH3 binding domain, HepG2 cells were transfected with wild type and mutant HBV genome cloned in pcDNA3.1 vector. The comprehensive protein profile indicating global cellular events in response to transfection with HBV genome was in turn analyzed by the high throughput, broad-spectrum and sensitive iTRAQ-coupled LC-MS/MS analysis. Our results suggested that the iTRAQ LC-MS/MS based quantitative proteomics analysis is an effective approach to investigate the comparative protein profile in HepG2 cells under HBV genome expression. A series of differentially expressed proteins were identified, some of which were highly related with cell migration. The integrated effects of these differentially expression proteins may provide valuable information on the underlying mechanism of the function of HBx in HBV-associated HCC and the specific interaction between HBx and vinexin- β [1].

Chapter 1 Introduction

1.1 Cell adhesion and deadhesion on thermo-responsive polymer (TRP) surface

Poly(N-isopropylacrylamide) (PIPAAm) emerges as the most popular thermo-responsive polymer (TRP) and is widely used in biomedical research. For example, PIPAAm has been widely applied in cell sheet regeneration and recovery. Recently, PIPAAm has been utilized as an effective biomaterial for developing co-culture system of several cell types. In general, free PIPAAm is insoluble in water above the low critical solution temperature (LCST, approximately 32°C) due to the formation of colloidal aggregate between the hydrophobic chains of PIPAAm. Interestingly, PIPAAm becomes reversibly soluble in water below the LCST due the increase of hydrophilicity of PIPAAm chains. This inverse temperature phenomenon of PIPAAm (Figure 1.1), i.e. hydrophobic above LCST and hydrophilic below LCST, was first reported more than thirty years ago. The unique temperature-dependent characteristic has only been utilized for various biomedical applications. Much attention has been paid to the development of thermo-responsive and cell-adhesive material on which cells adhere, spread, migrate, and proliferate well at physiological temperature. It is because conventional material like tissue culture grade polystyrene (TCGP) only

allows the invasive recovery of confluent cell layer with the use of trypsin. Thus all the essential cell membrane proteins are subjected to disruption by trypsin. With the use of TRP as a support for cell culture, adherent cells are de-adhered, detached and recovered with the use of any enzymatic treatment at room temperature.

Figure 1.1 The inverse temperature phenomenon of PIPAAm-grafted surface.

In general, the entire process of cell adhesion onto biomaterial surface can be commonly described by a two-step mechanism. First of all, passive adhesion is driven by a complicated partnership of various intermolecular forces including hydrophobic, electrostatics and van der Waals forces between the cell membrane and the opposing surface. Secondly, active adhesion which is directly triggered by ligand-receptor interaction and subsequent metabolic processes leads to the firm attachment of cell onto the biomaterial surfaces. In similar to any case of cell adhesion, cell deadhesion from PIPAAm-modified dishes is triggered by both

passive and active processes as mentioned above. Above LCST, ECM proteins from culture medium preferably adsorb on the hydrophobic PIPAAm surfaces. The passive process of cell deadhesion is directly induced by the alteration of surface properties of PIPAAm from slightly hydrophobic to highly hydrophilic during the reduction of temperature across the LCST. It is widely believed that the desorption of ECM proteins from PIPAAm surface below LCST leads to the deadhesion/detachment of cells. Otherwise, these ECM proteins adsorbed on the substrate and cell receptors on adherent cells are readily degraded during cell deadhesion by dispase (e.g., trypsin) treatment during conventional cell recovery.

The active mechanism of cell deadhesion towards temperature reduction is likely related to ATP-dependent cell metabolism [1]. Recently, it has been shown that active energy-dependent process is mediated by intracellular signal transduction, including cytoskeleton reorganization and tyrosine phosphorylation, leading to morphological changes of from highly spread to round after the change in the surface property [1, 2]. Furthermore, traction and contractile forces exerted by the cytoskeleton of cell adhering to the substrate are observed in viable cells. It is shown that cell traction is generated by the cytoplasmic termini of the cytoskeleton connecting to the intracellular domains of ECM receptors, such as integrins. These mechanical forces have been shown to detach coated ECM such as fibronectin from the tissue culture dish surfaces [3]. To validate the effect of cell metabolisms on cell deadhesion, sodium azide, a known inhibitor of cytochrome C

oxidase in mitochondria which reduces ATP generation, is loaded on cardiac myocyte sheet and hepatocyte sheet during deadhesion [1, 2]. Sodium azide was shown to delay the cell deadhesion and to inhibit the shrinkage of cardiac myocyte sheet. The result clearly indicates that ATP-dependent metabolic process is one of the major mechanisms responsible for cell sheet deadhesion from PIPAAm-grafted surfaces. If the cells are incubated for additional 5 min at 25°C after 30 mins treatment at 22 °C, the cell deadhesion will be accelerated. It is because the higher temperature accelerates the ATP generation [1, 4].

The behavior of cell sheet deadhesion is also correlated with cell types. Different cell lines also exhibit different behavior towards the temperature transition on PIPAAm. For hepatocyte, the optimum temperature for cell deadhesion is 12 °C and is significantly lower than that of endothelial cells (22 °C). This is another evidence to support hypothesis of active cellular metabolisms are necessary for effective cell deadhesion from TRP surface. On the other hand, the physiochemical properties of TRP are sometimes not optimized for cell adhesion. To improve biomaterial performance, the introduction of specific functional groups which directly promotes cell adhesion through the design of PIPAAm copolymer system is necessary. Moreover, the coating of ECM proteins on PIPAAm surface is an alternative method to enhance cell adhesion on biomaterials. ECM proteins, such as collagen, fibronectin, laminin, are widely used to improve the bio-specificity of the polymer surface towards targeted cells. The two methods of

biomaterial modification as mentioned above are crucial for TRP design for specific applications.

It is well known that protein adsorption on biomaterials affects cell spreading and adhesion. For instance, the adsorption of fibronectin and collagen drastically enhanced cell spreading, while albumin adsorption which has been used as negative control reduced cell adhesion. On pattern TRP-grafted TCPS surface, albumin selectively adsorbs on the non-grafted regions below LCST. Subsequently, fibronectin adsorbs on the region with grafted TRP above LCST [5]. The result indicates that STO fibroblasts adhere and spread on the entire surface with either coated fibronectin or albumin above LCST. When the temperature is reduced below the LCST, cells cannot be detached from fibronectin-coated region. The result strongly indicates the formation of strong adhesive interaction between the fibronectin layer and cell surface since the acute increase of surface hydrophilicity doesn't lead to the desorption of the fibronectin layer from TRP-grafted region [5].

In another study, bovine aortic endothelial cells were cultured in medium with serum on PIPAAm-grafted TRP surfaces [6]. The cells recovered from TRP surfaces maintained cell-cell junctions within the monolayer of cell sheets. It has been shown through immunoblotting and immunofluorescence microscopy that fibronectin (FN) was deposited and accumulated on the PIPAAm grafted surfaces above LCST during cell culture. Furthermore, the deposited FN matrix adhering to

cell sheets was also recovered from TRP surfaces by low-temperature treatment, while the ECM matrix was destroyed by trypsin treatment. Interestingly, the recovery of FN by low-temperature treatment was as high as that by physical scraping with a rubber blade. When hepatocytes fully spread and form significant adhesion contact on FN-coated PIPAAm surfaces, FN will be detached together with cell as detected by immuno-staining of fibronectin [3]. In the PIPAAm area without cell spreading and attachment, FN adsorbed remained to be surface-bound after the reduction of temperature below the LCST. The result indicates that active processes of adherent cells and hydration of PIPAAm layer are both required for the effective release of FN from the surface [3]. If collagen is coated on PIPAAm, hepatocytes also adhere on the substrate, and that viable cells are easily recovered by lowering the temperature regardless of the presence of collagen [7]. In fact, various proteins such as immunoglobulin, serum albumin, myoglobin, and lactalbumin were adsorbed onto PIPAAm surface above LCST, and triggered to desorb from PIPAAm after the temperature was reduced below the LCST. Until now, the effect of ECM protein coating on the deadhesion mechanism of single cells or cell sheet from TRP hasn't been systematically studied.

Based on the important role of active processes in cell adhesion and deadhesion, the cytoskeleton properties of cells would likely affect the intricate mechanisms of cell deadhesion from TRP. For instance, actin filaments provide the

mechanical support for the cell, dictate cell shape and drive cell migration (through lamellipodia, filopodia, or pseudopodia) Moreover, actin participate in the formation of intracellular junctions, in cytoplasmic streaming and in contraction of the cell during cytokinesis. Along with myosin, actin plays an essential role in muscle contraction [8, 9]. Normally, the F-actin resists mechanical stress and concentrates at the periphery of adherent cells. During cell deadhesion from PIPAAm, single cells can still maintain the peripheral rings of actin. Peripheral actin bands composed of actin filaments are known to exert contractile forces and to shrink the whole cell sheets after deadhesion. Since actin is not damaged by low temperature treatment, cytoskeleton induced contraction plays a critical role in the deadhesion process of sparsely plated cells and cell sheet from TRP [10].

In my thesis, PIPAAm grafted glass coverslip is used as a model surface to exploit the effect of HBV replication on the deadhesion kinetics of adherent cells.

1.2 The impact of HBV infection on the cellular activity

1.2.1 Structure and genome organization of HBV

The HBV genome is composed of partially double-stranded circular DNA. The full length (–) strand and short length (+) strand of HBV genome are respectively 3020~3320 bp and 1700~2800 bp in length [11]. The non-coding and

(–) sense strand is complementary to the viral mRNA. After the transfection with HBV genome, the viral DNA is commonly found in the cell nucleus. The partially double-stranded DNA get fully double-stranded through complete synthesis of the (+) sense strand through the removal of a short sequence of RNA from the (+) sense strand and the elimination of a protein from the (–) sense strand. Non-coding bases are also discarded from the ends of the (–) sense strand thus both ends are rejoined.

There are four known genes including *C*, *X*, *P*, and *S* encoded by the HBV genome (Fig. 1.2). The core viral protein is encoded by gene *C* (*HBcAg*), whose start codon is preceded by an upstream in-frame AUG start codon by which the pre-core protein is produced. After proteolytic processing, HBcAg is generated from the pre-core protein. The viral DNA polymerase and surface antigen (HBsAg) are encoded by gene *P* and gene *S*, respectively. The *HBsAg* gene contains a long open reading frame (ORF) as well as three in-frame "start" (ATG) codons which divide the gene into three sub-sections including *pre-S1*, *pre-S2*, and *S*. Due to the multiple start codons, polypeptides of three different sizes known as large, middle, and small (*pre-S1* + *pre-S2* + *S*, *pre-S2* + *S*, or *S*) are produced [12]. The function of the protein HBx encoded by gene *X* remains obscure [13].

Hepatitis B virus genome organisation

Figure 1.2 HBV genome organization.

The inner circle represents the virus genomic DNA that is packaged within viral particles in the cytoplasm of infected cells. The outer circles represent open reading frames (ORF) corresponding to core (C), surface antigen (S), polymerase (P), and HBx (X) proteins.

1.2.2 Hepatitis B virus X protein (HBx)

HBx is a 154-unit amino acid, with a molecular weight of approximately

17.5 kDa. Due to the lack of experimental data from X-ray crystallography (HBx has defied high-resolution crystallization) and nuclear magnetic resonance, little is known regarding the three-dimensional structure of HBx. Most studies have shown that HBx is mainly found in the cytoplasm of cells but it is also detected in the nucleus. More recently, it has been reported that HBx predominantly locates in the nucleus in cells when it is expressed at very low levels. However, HBx is dominantly found in cytoplasm as its expression level increases [14].

The exact function of HBx in HBV infection remains a long-term debate. HBx has shown no effect on viral replication in Huh7 hepatoma cell line and is inessential in the viral replication in transgenic mice, however it was also shown the ability of enhancing the replication of virus via the intermediate of other HBV gene expressions [15-17]. On the other hand, in the woodchuck model, HBx is required for persistent infection [18, 19]. Similar result has been recapitulated in human hepablastoma cell line HepG2 [20-22].

Although these studies only involved HBx expression instead of the replication of the whole HBV genome, a dynamic distribution of HBx could be cardinal, considering the multiple functions of HBx during the HBV life cycle. It has been widely known that HBx expression influences the transcriptional activation in the nucleus and viral replication in the cytoplasm.

1.2.3 HBx and cellular signaling pathways

HBx has been proven to stimulate the transcription of a series of viral and cellular transcription factors [23]. It increases viral genome expression and replication through the transactivation of cellular promoter and enhancers critical for persistent viral infection [24, 25]. Integration of the HBx sequence into host genome is a common event in HCC. After integration, HBx augments genetic instability via a variety of mechanisms, whereas undermines nucleotide excision repair through the inactivation of the UV-damaged DNA binding protein, the inactivation of p53, and interference with the association of p53 to excision repair cross complementing factor 3 and the transcription factor IIIH transcription factors, XPB and XPD [26, 27].

Several cytoplasmic signaling pathways, including activator protein-1 (AP-1), AP-2, JAK/STAT, nuclear factor- κ B (NF- κ B), PI3K, protein kinase C (PKC), ras-raf-MAPK, Smad, stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) and Wnt, have been reported to be transactivated by HBx through the combination to various nuclear transcription factors, including activating transcription factor 2 (ATF-2), cAMP response element binding (CREB), Oct-1 and TATA box binding protein (TBP) [25, 28].

Figure 1.3 Cellular signaling pathways involved in HBx-mediated hepatocarcinogenesis [29].

Bolded boxes denote key driving mechanisms underlying carcinogenesis.

HBx expression activates the Ca^{2+} -dependent proline-rich tyrosine kinase 2 by enhancing cytosolic calcium levels [21, 30], resulting in the activation of Src kinase [31]. Src strengthens the association of Shc-Grb2-Sos, afterwards activates Ras and the Ras-Raf-ERK/Ras-MEKK-JNK cascades and thus elicits the transcription of genes of the AP-1 family including c-fos and c-jun [30, 32]. It has also been proven that HBx activates NF- κ B in a Ras-dependent fashion [33].

The proteasomal degradation of β -catenin has been shown to be reduced by HBx, while HBx expression in HCC tissue is significantly correlated with β -catenin accumulation [34]. The downstream targets of β -catenin including c-myc and cyclin D-1 are also increased by HBx. The interaction between a serine-proline motif of HBx and Pin1, a Wnt signal regulator, has also been

reported. The up-regulation of Pin1 enhances the stability of HBx, whereas co-expression of Pin1 promotes HBx-mediated transactivation [35].

HBx represses the transcription of the *p53* gene [36]. Through interaction with p53, HBx inhibits several essential p53-dependent activities, including DNA repair, cell-cycle regulation, apoptosis and tumor suppression [37].

HBx up-regulates TGF- β , a mediator of fibrosis and apoptosis, in HCC tissue [38]. The phosphorylation of Smad3 at its C-terminal regions and/or middle linker is critical for TGF- β signaling. Smad3 phosphorylated at the C-terminal region (pSmad3C) or the middle linker (pSmad3L) transmits a tumor-suppressive or oncogenic TGF- β signal, suggesting a reversible shifting between tumor suppression and oncogenesis [39]. In the early carcinogenic stage, the hepatocytic TGF- β signaling is shifted by HBx from the tumor-suppressive pSmad3C to the oncogenic pSmad3L pathway [40]. It has been reported recently that the paracrine activation of human HSCs is induced by HBx whereas blocked by anti-TGF- β [41].

It has been reported recently that HBx induces steatosis, a key cofactor for chronic liver disease and HCC, through activating the transcription of SREBP1 and PPAR- γ [42]. HBx induces liver X receptor-mediated lipogenesis in HCC [43, 44].

Inhibition of Fas-mediated apoptosis [45], induction of genetic instability via binding to damaged DNA binding protein 1 [46], modulation of the degradation of cellular and viral proteins through inhibition of serine protease inhibitors and proteasome complexes [47], induction of oxidative stress and mitochondrial translocation of Raf-1 kinase [48], up-regulation of insulin receptor substrate-1 [49], up-regulation of beclin 1 and a corresponding increase in starvation induced autophagy [50, 51], interaction with DNA methyltransferase (DNMT) with abnormal epigenetic modifications [52, 53] and survival benefit under oxidative stress [54]: all above reported process are potential mechanisms of HBx-mediated hepatocarcinogenesis.

1.3 Proline-rich region in SH3 binding domain of HBx and SH3-mediated signaling

It is critical to clarify protein-protein interaction for the comprehension of the cellular signal transduction. The interactions can be identified and characterized by various techniques including cDNA library screening, yeast two-hybrid, co-IP (immunoprecipitation), mutagenesis and truncation. Protein-protein interactions can occur through noncatalytic, conserved small domains as short as 10 amino acid residues in a single protein. Among the primary sequence of the interacting motifs, proline is a fairly special one. Significantly, the

well defined SH3 domain, WW domain and several other domains specifically recognize ligands which are rich in proline residues.

SH3 (Src Homology 3) domain, part of the Rous sarcoma oncogene product, plays a vital role in signal transduction. SH3 domain is typically a small, conserved sequence with 60 amino acids. It interacts with proline-rich binding signature motifs known as SH3 binding domains. These domains usually consists of 6 to 12 amino acids, with a conserved Proline-X-X-Proline (PXXP) motif [55], where X denotes any single amino acid.

SH3 domains can be considered as an adaptor molecule, functioning by attracting downstream proteins in a signaling pathway cascade. They facilitate the assembly of signaling complexes, such as epidermal growth factor receptor signaling [56], cellular localization of cytoplasmic proteins [57], and activation of PI3K in response to IgM crosslinking [58]. In the context of viral infections, SH3 domains are also believed to be pivotal links between the virus and the host cells. Furthermore, SH3 domains have been implicated in cancer development. It has been reported that Nef, the HIV viral protein, interacts with the p21-activating kinase 1 (PAK1) via its SH3 binding domain and viral replication is enhanced as a result. It is also believed that NS5A, non-structural protein of HCV, interacts through its C-terminal PXXP motif with the cellular linker protein Grb2 [59], resulting in a reduction of the mitogenic signaling pathway and inhibition of the

activating protein 1 (AP-1). As for HBV, it has been shown that HBx activates SH3 domain containing signal protein c-Src and the activation although no direct protein-protein interaction motif has been identified [60]. As a result of the wide range of interacting targets, the SH3 domain holds the great promise in the finding of new therapeutic agents that are particularly specific to a certain signaling pathway. SH3 domain act could be inhibited by the dissociation of its binding activity [61, 62].

Our colleagues have recently reported by SH3 Domain Arrays that HBx contains a proline/serine motif which appears to interact with a number of SH3-binding cellular proteins [63].

1.4 The correlation between HBV infection and cell migration

Hepatocellular carcinoma (HCC), one of the most prevalent malignant tumors in Asia, has frequently led to terminal complications of chronic inflammatory and fibrotic liver disease [64]. The mortality rate of HCC is significantly high due to its high metastasis rate. HBx, a small regulatory protein with 154 amino acid residues is required for viral infection. Also, it has been reported that HBx interacts with a wide range of cellular proteins [65] and plays an essential role in the development of HCC [66, 67]. Nevertheless, the molecular mechanism of HBx-mediated carcinogenesis remains to be determined. It has been

shown that HBx plays various biological roles, including transcriptional activator of numerous viral and cellular promoters. For instance, HBx has been reported to be implicated in the disruption of host DNA repair, modulation of apoptosis, inhibition of physiological proteolysis, induction of cancer cell migration and interaction with p53 [13]. Another example is the acquisition of metastatic HCC which is frequently observed at the early stages of HCC and may cause liver failure and ultimately death [68, 69]. Taken together, the initiation and progression of HCC correlated with hepatitis B virus (HBV) infection may attribute to these underlying mechanisms.

An important molecular mechanochemical sensor which anchors between cell and extracellular matrix (ECM) is the integrin receptor. Integrin interacts with cytoskeletal proteins through its intracellular domain and binds to ECM molecules with its extracellular domains. It is known that HBV infection can disrupt the integrin-mediated signaling cascades [70]. For instance, HBx blocks the molecular linkage between the actin filament and cadherin complex and weakens the intercellular adhesion in a Src-dependent manner [71]. Moreover, HBx decreases the expression of $\alpha 1$ and $\alpha 5$ subunits of integrin, impairs cell adhesion on fibronectin coated substrate and promotes cell migration [72]. Consequently, it is generally anticipated that the interaction between HBx and actin-binding protein influences the morphological changes of hepatocytes through the alteration of the cytoskeleton organization. On the other hand, little is known on the intricate effect

of HBV replication on the biophysical mechanisms of cell–ECM interactions.

The invasion and metastasis of cancerous cells is a multi-step process [73]. HCC frequently invades blood vessels in early stage and metastasize intra-hepatically and later extra-hepatically [74]. Meanwhile, the degradation of the extracellular matrix (ECM), including the basement membrane, is a critical initial step in cancer metastasis. The normal architecture of ECM is critical to the regulation cell homeostasis and proliferation. To invade or metastasize, cancerous cells have to first overcome a mechanical and physical barrier consisting of the surrounding ECM and the encapsulating basement membrane [75, 76]. Destruction of the ECM by MMPs has been shown to play an important role in the enhancement of migration and spreading of tumor cells, resulting in tumor invasion and metastasis. A permissive microenvironment plays an essential role in the determination of the fate of tumor cells, ultimately tumor progression and metastasis.

It has been proven in previous studies that matrix metalloproteinase-2 (MMP-2), a 72-kD type IV collagenase, might play an essential role in HCC invasion and metastasis [77]. It has also been reported that there is a strong correlation between the induction of membrane-type 1 matrix metalloproteinase (MT1-MMP) and MMP-2 and HBx expression in HCC patients, leading to poor prognosis. Recent experimental finding has demonstrated that HBx induces

overexpression of MT1-MMP, subsequently activating MMP-2. Moreover, the MMP activation induced by HBx is found to be correlated with the enhancement of cell migration. The result strongly supported the role of HBx in HCC invasion and metastasis. Although the chronic HBV infection has been acknowledged as a major cause of HCC, the mechanisms of HBV-mediated carcinogenesis remains to be elucidated [78]. Regularly detected in HBV carriers, HBx has actually been nominated as the most prevailing biomarker of HBV infection among commonly expressed HBV proteins [79]. Additionally, HBx is often found as the sole HBV protein expressed in HBV-infected hepatocytes [80]. A positive HBx expression, which was closely correlated with the overexpression of MT1-MMP and MMP-2, was demonstrated clinically in 80.1% of samples taken from 136 HCC patients. In one word, the consistence between HBx expression and overexpression of MMPs is the hallmark of a poor prognosis.

Indeed HBx was shown to promote the expression of MT1-MMP through the introduction of HBx protein into a hepatocyte line such as Chang cells [80, 81]. Interestingly, it has also been demonstrated that HBx-mediated up-regulation of MT1-MMP elicited the activation of MMP-2. The result is accordant with the role of MT1-MMP as an upstream regulator of MMP-2. MMP activation could accelerate ECM degradation, disrupt the physical barriers composed by the ECM and thus enhance cell migration. Furthermore, it has been validated that a MMP inhibitor completely counteracted the functions of HBx [82, 83]. Taken together,

all results as mentioned above indicates that cancerous cells expressing HBx reveal a remarkably enhanced cell migration through the activation of MMPs.

It has been found the infection of HBV increases the invasiveness of HCC cells through the transcriptional activation of Matrix metalloproteinase-2 (MMP-2), MMP-3 and MMP-9 [81-83]. HBx has been proven to enhance the transcription, translation and secretion of matrix metalloproteinase-9 and metalloproteinase-3[84] which affect cell adhesion and migration [85]. Recent studies also suggest the possible role of HBx in cell apoptosis, a process involving major cytoskeletal disruption [86].

Dysregulation of cell adhesion and uncontrolled high motility are hallmarks of tumor development and metastasis. Metastasis is a major cause of death among cancers. A number of proteolytic enzymes participate in the processes of cell invasion and metastasis to degrade noticeably the extracellular matrix (ECM) [87]. Thus, the abnormal cytoskeletal reorganization could be a sign of tumor development and cell metastasis.

HBx is also found to induce cell migration in a CD44-dependent manner [88]. Such activation is mediated by PI-3K-Akt/ protein kinase B (PKB) and extracellular signal-regulated kinase 1/2 (ERK 1/2). In addition, altered Rho GTPase signaling is implicated in the initiation and the progression of HCC [89].

Rho downstream effector Rho kinase 2 (ROCK2) is frequently over-expressed in HCC and the knockout of ROCK2 influences the formation of stress fibers, filopodia, and lamellipodia [90].

1.5 The modulatory behavior of EGCG in cancer development

Green tea (*Camellia sinensis* leaves) is one of the most prevalent beverages worldwide. The consumption of green tea is regarded as benefit to health and anti-aging. Regarding cancer development, it is generally acknowledged that the chemopreventive effects of green tea are mainly mediated by its polyphenols. Among the major catechins in green tea including epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate, epigallocatechin and epicatechin, EGCG is the most abundant component and accounts for 50~80% of the total dry weight. The chemical structure of EGCG, as shown in Fig. 1.4, can also be produced by stereoselective total synthesis [91].

Figure 1.4 Chemical structure of EGCG [91].

EGCG modulates cellular signaling in various ways. It has been reported that EGCG induces apoptosis and cell cycle arrest in many cancer cells [92-94]. Moreover, the inhibition of nuclear factor- κ B signaling pathway [95-97], MAPKs, activator protein-1 [95, 97, 98], epidermal growth factor (EGF) receptor-mediated signal transduction pathway [97, 99, 100], insulin-like growth factor-I (IGF-I)-mediated signal transduction pathway [101], overexpression of cyclooxygenase-2 [102, 103], and proteasome activity [104] have been proven to be inhibited or decreased by EGCG, suggesting its cancer-preventive effect.

EGCG has also been targeted as an active modulator of cell signaling associated with angiogenesis, oncogenesis, and especially cancer cell migration and metastasis. In EGCG-treated cells, vascular endothelial growth factor (VEGF) receptor phosphorylation was found to be decreased [105, 106]. A combination of EGCG with ascorbic acid was also implicated in the inhibition of cancer cell

proliferation and migration [107].

Several members of the matrix metalloproteinase (MMP) family including MT1-MMP, MMP-2 and MMP-9, were shown to be remarkably inhibited, inactivated or repressed by EGCG treatment in TRAMP mice [101], endothelial cells [108], breast cancer cells [109], lung carcinoma cells [110], glioblastoma cells [111] and fibrosarcoma cells [112]. It has been widely recognized that MMPs are commonly overexpressed or up-regulated in various cancer cells [113, 114] and are involved in tumor development and invasion through the destruction and turnover of extracellular matrix [115, 116].

The anticancer activity of EGCG has been further validated by its inhibition of urokinase-plasminogen activator (uPA), one of the most frequently overexpressed enzymes in human cancer cells [117]. EGCG has also been implicated in the down-regulation of ephrin-A1-mediated endothelial cell migration [118] as well as melanoma and pancreatic cancer growth and metastasis [119, 120]. It has been demonstrated that a co-treatment of prostate carcinoma cells with TNF-related apoptosis-inducing ligand and EGCG led to a decreased cell migration [121].

Till now, most studies of EGCG focused on its impact on proliferation, apoptosis and modulation of multiple signaling pathways. Moreover, most studies

investigating cell migration were performed by applying *in vitro* platforms and based on qualitative rather than quantitative data. Whereas our approaches aiming at contact kinetics and cell motility provides a novel quantitative perspective. Moreover, many results regarding the modulatory roles of EGCG in cell migration were reported in various carcinoma models including lung cancer, breast cancer, prostate cancer and gastric cancer [109, 122-126]. The quantitative work based on hepatocellular carcinoma remained rare.

A human liver carcinoma cell line, HepG2, was employed in this study as a liver cancer cell culture model system. Since cell adhesion, deadhesion and their dynamic assembly, migration, are fundamental activities and essential mechanisms underlying cancer invasion and metastasis, we investigated the consequence of EGCG treatment on cell adhesive, deadhesive and migratory behavior in order to develop new approach to evaluating therapeutic effect of EGCG. Firstly, HepG2 cells were treated with different concentrations of EGCG. Then the adhesion contact kinetics and cell geometry of various types of HepG2 cells during both adhesion and thermally triggered deadhesion were probed with the application of the confocal reflection interference contrast microscopy (C-RICM). Additionally, the immunofluorescence microscopy was applied to investigate the structural transformation of actin of HepG2 cells. Furthermore, the time-lapse fluorescence microscopy and a two-dimensional (2D) model of cell dispersion were employed to simulate conditions similar to those observed *in vivo* during tumor invasion in

order to explore the cell migration profile under EGCG treatment.

1.6 iTRAQ coupled LC-MS/MS based Proteome profiling

In the last decade, the demand of systematic approach to discover new biomarkers of cancers has arisen. The main task is to discover and validate biosignatures for the classification and identification of cancers. Taking into account the outcome of clinical diseases, these signatures can be correlated with the treatment outcomes. The biomarkers, including genomic changes, polymorphisms and proteomic changes, can provide guide to to diagnose disease and develop the treatment for patients with similar or analogous biomarker patterns after an appropriate validation. A number of techniques allow the testing for proteins produced during a particular disease, which helps to diagnose the disease quickly. In addition, biomarkers are useful in the detection of early stage cancer development by investigating differential protein expression or modification in healthy or impaired tissues from patients.

Proteomics is the large-scale study of proteins [127, 128]. The proteome of a cell is a complicated and dynamic entity altering with different physiological states and extracellular environments. Compared to the transcriptome in DNA microarray, the proteomics analysis aims to understand the end state production of specific proteins in response to the external stimuli. In the context of

HBV-associated liver diseases, proteomics has recently been applied for the understanding of the disease states such as cirrhosis and especially hepatocellular carcinoma [129].

In proteomics research, the comparative quantification of differences between several correlated samples or physiological states based on an identical organism or tissue is a critical but challenging technical task.

Conventionally, two-dimensional electrophoresis (2DE) is a widely used approach to separate proteins according to isoelectric point followed by molecular weights. This technique often generates the first screening protocol for the proteomics. It was first introduced by O'Farrell and J. Klose in 1975. This technique allows a resolution of 1000s of proteins and has since been refined to generate highly reproducible proteome maps by the emergence of immobilized pH gradient (IPG) gel strips.

The 2-D gel based proteomics analysis has been useful in the early stage of the identification of biomarkers as well as drug discovery. New development of dyes, fluorophores have led to improvements in sensitivity and dynamic range associated with 2-D gel based proteomics analysis. Nevertheless, the shortcomings of 2-D gel based analysis are becoming apparent. First of all, the higher resolution of protein separation is required. In addition, 2-D gel-based analysis alone is

unable to reveal the identity of underlying protein. Furthermore, the reproducibility of protein spots on 2D gels remains time consuming. These disadvantages can be overcome by the application of the increasingly popular LC-MS/MS approaches.

The LC-MS/MS approaches are based on mass-spectrometry quantification techniques. Generally, the cells or tissues targeted to be analyzed are firstly purified and the purified proteins were broken into peptides through gentle trypsinization, and the peptides were in turn applied to MS analysis as shown in Fig. 1.7.

Figure 1.5 Common quantitative mass spectrometry.

Recently, stable isotope labeling chemistries combined with MS have been developed. Differential stable isotope labeling has been used to generate a specific mass tag which can be recognized by the mass spectrometer during the process of quantification. These tags include isotope coded protein labels (ICPL), isotope-coded affinity tag (ICAT), stable isotope labeling by amino acids in cell culture (SILAC) and isobaric tagging and relative quantification (iTRAQ) [130]. ICAT method involves derivatization of cysteine residues with a reagent containing eight zero or eight deuterium atoms, followed by MS analysis. One of the advantages of ICAT is that since cysteine is a rare amino acid, such a label method can reduce the complexity of samples. ICAT is not appropriate for the proteins that do not have cysteines. In addition, it also has limitation in the analysis of post-translational modified proteins.

Another widely used approach for proteomics and biomarkers researches is the iTRAQ method [130]. iTRAQ, short for isobaric tags for relative and absolute quantification, has been used for the identification of cancer and other biomarkers of diseases [131-137]. The complete molecule consists of two parts, isobaric tag and reactive group. The part of isobaric tag is composed of balance group and reporter group. The total mass of the isobaric tag is 145. Specifically, the mass of reporter group is from 114 to 117, while the mass of balance group is from 31 to 28, which is used to maintain the total mass of 145 (Fig. 1.8). The current commercial multiplex iTRAQ reagents contain four or eight labels in the kit providing the

labeling of up to four samples or eight samples in one experiment respectively. The protein samples are digested into short peptides by gentle trypsinization and fragmented peptides are labeled with different isobaric tags. The labeled peptides was then mixed and analyzed in a tandem mass spectrometer. During the fragmentation by tandem MS, the balance group and reporter group is separated by cleaving the bond between them and only the balance group remain binding to the peptide. Meanwhile, the released reporter groups can send a defined MS signal. Since all reporter groups of isobaric labels share the same physicochemical parameters excepting the mass [133], the differential abundance of the reporter groups are proportional to the separately labeled peptides from each sample.

In the tandem MS analysis, with a reagent labeled, the peak area in the spectrum is proportional to the relative abundance of the peptide which is also proportional to the original protein quantity. Four differentially labeled samples were mixed first and after application on MS/MS, the relative concentration between different isobaric reporter ions can be acquired to reveal the peptide abundance in different samples. Fig 1.8 shows a typical Mass spectrum of a single peptide prepared by labeling four separate digests with each of the four isobaric reagents, and combined the reaction in 1:1:1:1 ratio. In ii) of Fig. 1.8, the four isobaric labels show the same area of the peak which is consistent with the ratio of abundance of the labeled peptide and mixture.

Figure 1.6 The components of the multiplexed isobaric tagging chemistry [130].

Figure 1.7 Example of a typical MS/MS spectrum [130].

A typical MS/MS spectrum of one peptide. After digestion, the peptide was labeled separately with four isobaric reagents at the ratio of 1:1:1:1. The labeled peptides were mixed together and analyzed by the LC-MS/MS [130].

Gas chromatography and mass spectrometry (GC/MS) have been extensively used to identify and quantify relatively small molecules (with

molecular weight less than 1,000 Da). Over the last decade, this technique has been increasingly used for the comprehensive analysis of cellular components. Development of new technologies have been reported to effectively volatilize and ionize macromolecules, using various matrices and lasers (MALDI) or electrospray ionization (ESI) [138]. High resolution of spectra display is offered by measuring the accurate mass-to-charge ratio (m/z) [137]. The tandem mass spectrometry facilitates data acquisition (protein sequence) and transformed the field of proteomics [137]. It has been further strengthened by the coupling of mass spectrometers with high performance liquid chromatography (HPLC) [137]. In MS/MS, precursor ions are labeled in full-scan mode (all m/z values) [137]. This is followed by selective ion isolation and fragmentation for sequence identification [137]. Ions generated in solution from ESI make them suitable for coupling to LC. In addition, a reverse-phase HPLC setup allows the column to slow down the migration of peptides based on selective hydrophobic interaction affinities [137]. Peptides can then be eluted with a gradient of organic solvent, such as acetonitrile. They are then ionized before being injected into the mass spectrometer. In general, the quality of proteomics profiling needs to be sensitive, display wide coverage, possess efficient protein fragmentation, shows high mass resolution and reproducible [137]. These requirements have been achieved by LC-MS, as femtomole or even lower detection limits can be reached even in the setting of complex cellular mixtures.

Chapter 2 Materials and Methods

2.1 Preparation of thermo-responsive cell culture surface

2.1.1 Preparation of PIPAAm-grafted surfaces

Glass coverslips were cleaned with Piranha solution (30% v/v H₂O₂ and 70% v/v H₂SO₄) for 1 hour, rinsed thoroughly with doubly distilled water (resistance: 18 MΩ, Sartorius, Germany) and then dried in vacuum oven at 22 °C. The preparation of PIPAAm-grafted surfaces using the atom transfer radical polymerizations (ATRP) reaction have been previously depicted in detail [139]. In brief, glass coverslips were first immersed in 30 ml of chloroform (Sigma-Aldrich Inc., USA) followed by the addition of 0.5 ml of triethylamine (Sigma-Aldrich Inc., USA) and 2 ml of 4-(chloromethyl)-phenyltrichlorosilane (Alfa Aesar Pte. Ltd., Singapore). After 24 hours of reaction, the coverslips were thoroughly rinsed with acetone and dipped in acetone for another 30 min in order to remove the non-reacted silane. After being dried in air, the silanized coverslips were dipped into 15 ml of N-isopropylacrylamide (NIPAAm) (Sigma-Aldrich Inc., USA) solution with a concentration of 0.23 g/ml, followed by 20 mg of CuCl and 4 mg of CuCl₂ (Sigma-Aldrich Inc., USA). The solution mixture was degassed with argon gas for 20 min. Finally, 5 μl of 1,1,4,7,10,10-hexamethyl-triethylenetetramine (Sigma-Aldrich Inc., USA) was added to the mixture and the reactor was sealed with ParaFilm for the interfacial polymerization of NIPAAm. To stop the

polymerization, the glass substrates were taken out from the solution mixture and washed consecutively with abundant amount of DMSO (Sigma-Aldrich Inc., USA) and double distilled water (Sartorius, Germany), and then dried in vacuum oven. The root-mean-square layer thickness, roughness at 22 °C and 37 °C of PIPAAm-grafted surface are respectively 33 ± 5 nm, 1.7 ± 1.8 nm and 1.1 ± 0.8 nm, while the water contact angle at 22 °C and 37 °C are $61\pm3.1^\circ$ and $79\pm3.6^\circ$ as reported elsewhere [139, 140]. The XPS measurements were performed on a Kratos AXIS HSi spectrometer using a monochromatized Al Ka X-ray source (1486.6 eV photons). An atomic force microscope (Model: MFP-3D, Asylum Research, CA) with water-immersion capability was used for all nanotopographical measurements with contact mode under water at 37 and 22 °C, which is above and below the LCST of PIPAAm, respectively. A scanning rate of 1 Hz was used in all experiments. An electronic fluid cell was applied to control the temperature of the PIPAAm surface under water. Each AFM topographic image was representative of at least 10 images on three samples and is highly reproducible using the same procedures of sample preparation. In certain cases, AFM measurements of PIPAAm or ECM protein-coated PIPAAm surfaces were carried under the dry mode at 22 °C.

2.1.2 Preparation of laminin-coated PIPAAm surface

400 μ l of 0.1 mg ml⁻¹ laminin solution, which was diluted from the original laminin solution (Sigma-Aldrich Inc., USA, 1.0 mg ml⁻¹ in water) was directly

loaded onto the PIPAAm surface and incubated at 4 °C for 24 hours. The surface was then thoroughly washed with deionized water to remove the excess laminin. After being sterilized with 70% ethanol, the surface was stored in 4 °C refrigerator before further experiments.

2.2 Mutant HBV genome cloning constructions

2.2.1 Mammalian expression vector

The pcDNA3.1 vector is a widely used commercial mammalian expression. The organization of pcDNA3.1 vector was shown in the Figure 2.1. Our replicative HBV genomes were constructed in this vector. The successful expression can be validated by further RT-PCR of core gene and surface gene of HBV.

Figure 2.1 The feature map of mammalian expression vector pcDNA3.1.

MluI restriction site, prior to the pCMV promoter was used for cloning of the replicative HBV genome instead of the multi-cloning site (MCS).

2.2.2 Site-directed mutagenesis

To generate mutant constructs, site-directed mutagenesis was used (QuickChange Site-Directed Mutagenesis kit, Stratagene). The primers were designed according to the sequence flanking the target mutation sites. Short primers of 17~18 nucleotides were generally used. Following the instructions of the manufacturer, PCR was applied with the designed primers, the reagents, and the diluted (usually 1:50) template plasmid of interest. The applied reaction system was as follows: 5 μ l of 10 $\times$ reaction buffer (100 mM KCl, 200 mM Tris-HCl pH 8.8 and 100 mM (NH₄)₂SO₄), 1% Triton X-100, 20mM MgSO₄ and 1mg/ml

nuclease-free BSA), 125 ng of the forward and reverse primers each, 50 ng of dsDNA template, 1 µl of dNTP mixture and RNase-free water was added to top up the reaction mixture to be 49 µl. 1 µL of pfu Turbo DNA polymerase (2.5U/µL) was then added to the reaction mixture. The PCR reaction was carried out in this cycling conditions: 95°C 30 seconds; 18 cycling of 95°C 30 seconds, 55°C 30 seconds, 68°C 5 minutes (may vary depending on the size of the target plasmid with a speed of 1 kb/min). The completed PCR reaction should contain the newly synthesized plasmids with site mutations. The parental strands were removed by adding DpnI (1 µl) and an incubation at 37°C for 2 h. The products were transformed into *E.coli* cells provided in the kit as high efficiency competent cell DH5α. And the positive colonies were selected. The efficiency of site-directed mutagenesis was confirmed by sequencing the mutation site from a up-stream primer.

2.2.3 Preparation of mutant HBV genome

The design of DNA primers for mutation analysis of the genotype B SH3 binding domain from prolines to alanines within HBV genome is shown in Table 2.1. This set of four primers which was used to generate HBx mutated in the proline-rich region within full length HBV genome is cloned in pcDNA3.1+ vector with QuikChange® XL Site-Directed Mutagenesis Kit (QIAGEN, USA) (Table 2.1). “B mutant A” means the double mutated region A (P39 and P42), while “B mutant B” represents the double mutated region A (P43 and P46) of HBV genome

genotype B. After the two-step site-directed mutagenesis as described in 2.12, the mutant plasmids, wild type plasmids and empty vector were amplified by transformation into *E. coli* and then extracted using Plasmid Midi Kits (QIAGEN, USA) for cell transfection. The success of mutation generation was confirmed by DNA sequencing.

Table 2.1 Primer sequences of site-directed mutagenesis in proline-rich region of HBx.

B mut A sense (from 1443 bp to 1487 bp):

E S R G R **P** L P G **P** L G A L P
 5' GAA TCC CGC GGA CGA GCC CTC CCG GGG GCG CTT GGG GCT CTA CCG 3'
A A

B mut A anti-sense:

5' CGG TAG AGC CCC AAG CGC CCC CGG GAG GGC TCG TCC GCG GGA TTC 3'

B mut B sense (from 1491 bp to 1532 bp):

A L P P A S **P** **P** I V P T D H
 5' GCT CTA CCG CCC GCT TCT GCG GCT ATT GTA CCG ACC GAC CAC 3'
A A

B mut B anti-sense:

5' GTG GTC GGT CGG TAC AAT AGC CGC AGA AGC GGG CGG TAG AGC 3'

The two prolines (P) in translational amino acid sequences on *B mut A* sense and *B mut B* sense primer sequence were mutated into alanines (A).

2.2.4 Preparation of competent cells by CaCl₂ method

The preparation of LB broth, 2×YT broth and Agar plates were adopted from Molecular Clonings (Cold Spring Harbor Laboratory, 3rd Edition). The mixtures were autoclaved at 121 °C 20 min and cooled to 45~50 °C to the addition of required antibiotics. LB agar was then used to prepare agar plates by pouring onto the Petri dish. The broth and plates can be stored at 4 °C for several months.

Fresh agar plates were prepared without selective antibiotics. DH5 α or BL21 strains were streaked onto a fresh agar plate and cultured at 37°C for 12~16 hours. Single colony was selected from the agar plate and transferred into 5 mL LB broth without selective antibiotics and incubated at 37°C overnight with shaking at 220 rpm. The cultured bacteria was re-inoculated the next day into fresh 100 mL LB broth at 1:100 ratio and allow to grow to a concentration of OD₆₀₀ 0.3 to 0.4. The culture in the exponential phase was then aliquoted into 50 mL pre-chilled centrifuge tubes and incubated on ice for 10 min followed by centrifugation for 5 min at 5,000 rpm at 4°C. And the cell pellet was re-suspended in 30 mL of 0.1 M ice-cold MgCl₂ by vortex and pipetting. The thoroughly suspended mixture was then centrifuged for 5 min at 5,000 rpm under 4°C. The supernatant buffer was then decanted carefully and the pellet was re-suspended in 20 ml of 0.1 M ice-cold CaCl₂ and incubated on ice for 30 min and then centrifuged for 5 min at 5,000 rpm at 4°C. And again the pellet was re-suspended in 1.5 ml of ice-cold 0.1 M CaCl₂/15% glycerol. The prepared competent cells were then aliquoted in to 1.5 ml autoclaved and fresh opened Eppendorf tubes and stored in -80°C freezer for long term storage. All the manipulations were carried out in Biological Safety Cabinet since the no antibiotics was applied.

2.2.5 Plasmid transformation

Prepared and stored DH5 α or BL21 competent cells prepared as described

above were thawed on ice. 50 ng of plasmid DNA was added to the competent cells (around 100 μ L) followed with soft flicking and incubated on ice for 30 min. The mixture of competent cells and plasmid was then subjected for incubation for 90 sec at 42°C, a process named heat shock. This was quickly followed by incubation on ice for 10 min allowing the cool down. 800 μ L LB broth was added to the mixture and the cells were recovered by incubation at 37°C shaking at 150 rpm for 45 min. 100 μ L of the transformed cells were then plated onto prepared LB agar plate supplemented with selective antibiotics. The plate was then placed in 37°C incubator downside up until the all the liquid was absorbed by the LB broth and the plate was incubated overnight in an upside down position. The colonies, visible after an overnight incubation, were picked out from the plate and selection of positive transformation colony with insert was carried out as described below (2.2.6).

2.2.6 Plasmid amplification by QIAGEN QIAprep Miniprep

Plasmids of interest were transformed into the DH5 α strain designed for gene recombination and plasmids amplification. Single colony was selected and inoculated into 5 ml fresh LB broth with proper antibiotics followed by incubation at 37°C shaking at 220 rpm overnight. The culture was centrifuged at 5,000 rpm for 10 min to harvest the cell pellet. Supernatant was discarded and the bacteria cells

pellet was re-suspended in 250 µl ice-cod re-suspension Buffer P1 supplemented with provided RNase A (100 µg/ml). The bacteria were completely re-suspended by vortexing and pipetting up and down until no cell clumps was visible. 250 µl lysis buffer P2 was added and mixed thoroughly by gently inverting the tube 4-6 times. 350 µl neutralization buffer N3 was added and mixed 4-6 times gently and thoroughly. The mixture was then centrifuged for 10 min at a speed around 13,000 rpm (~17,900 g). Supernatant containing plasmids was then transferred into the QIAprep purification column without agitating the white pellet and subjected to centrifugation at 13,000 rpm for 30~60 s. The absorbed plasmids on the silica membrane were than washed by 500 µl buffer PB and 750 µl buffer PE followed by centrifugation of 30~60 s each time. An additional 1 min centrifugation was applied to thoroughly take away the residual washing buffer. The column with plasmids was placed into a new 1.5 ml EP tube and proper volume (30~ 50 µl) of distilled water or buffer Elution Buffer was added to the column. After incubation of 3 min, by 1 min centrifugation at 13,000 rpm, the plasmids were eluted from the membrane and dissolved in EB buffer or water. For high yield, second elution can be applied. Plasmid DNA collected by the elution was quantitated (see 2.9) and stored at -20°C until further use.

2.2.7 Plasmid amplification by Invitrogen Midiprep

Midiprep was applied to remove impurities such as endotoxin in DNA

preparation which may harm the mammalian cell after transfection. Invitrogen Midiprep kit was used for this purpose. Briefly, 50ml of transformed *E.coli* bacterial cell culture was collected after overnight growth by 10 min centrifugation at 10,000 g. The supernatant was discarded and pellet re-suspended in 3ml Cell Suspension Solution, followed by adding 3 ml Cell Lysis Solution. The mixture was incubated for 3 min at room temperature. 5ml Neutralization Solution was added to the lysed cells and mixed by gently inverting the tube 3~5 times. Another incubation of 2~3 min was added to allow a white flocculent precipitate to form. The lysate was then poured into PureYield Clearing Column (blue for lysate clearing) into a new 50 ml disposable plastic tube and incubate for 2 min. The column was centrifuge 1,500 rpm for 5 min. The PureYield Binding Column (white) was placed onto the vacuum manifold and the cleared lysate was poured into the white column. The lysate was forced pass through the binding column by applying maximum vacuum. The membrane bound plasmids were washed by 5 ml Endotoxin Removal and 20 ml Column Wash Solution and the vacuum was applied to draw the solution through. The membrane was made by applying the vacuum for 30 seconds. The DNA on the membrane was eluted by adding 600 μ l Nuclease Free water. The purified plasmids was then quantitated (see 2.2.8) and stored.

2.2.8 Plasmid DNA quantification by UV spectrophotometer

Plasmids DNA purified by QIAprep Miniprep or Midiprep was quantified

by UV spectrophotometry at OD_{260nm} and OD_{280nm}. The plasmid was diluted to proper concentration with distilled water at 125 ×. Pure water was used for zero calibration. DNA Yield was calculated by multiplication of by OD₂₆₀ 50 µg/mL and a further multiplication by the dilution factor. The formula is shown as following:

$$\text{OD}_{260\text{nm}} \times 50\text{ng} / \mu\text{l} \times 125 (\text{dilution factor}) = \text{Concentration ng} / \mu\text{l}$$

The purity of the plasmid DNA was obtained by dividing OD_{260nm} with OD_{280nm}. A value between 1.7 and 1.9 indicated a good preparation of the plasmid DNA.

2.2.9 Glycerol stock

One single colony was selected from the LB broth agar plate after transformation, and was inoculated into a 5 mL fresh LB broth with proper antibiotics followed by shaking at 220 rpm overnight at 37°C. The cultured bacteria were then mixed thoroughly with sterile glycerol with the ratio of 15~30 % in the sterile EP tube and all relevant data (host strain, vector, selection criteria, date prepared) were recorded on the tube. The tube was stored at -20°C. For long term storage, -80°C freezer was used. The glycerol stocked strain can be used for inoculation or recover by streaking.

2.2.10 Restriction enzyme digestion

For cloning purpose, single restriction enzyme or double restriction enzyme digestion were used according to the initial cloning strategy. Typically, the vector

plasmid DNA, PCR amplified product were digested by New England Biolab's (NEB) restriction enzymes. Enzymes were usually added in the concentration of 1 unit for each digestion reaction. 10 × reaction buffer was chosen to the appropriate enzymes to 1× in the final digestion mixture. For a number of enzymes, BSA was needed to optimize the digestion and 100 × BSA is provided by NEB. The mixture was incubated at 37 °C for more than 1 hour. Longer time of incubation was applied whenever appropriate. The digestion products were separated on agarose gel by electrophoresis and the DNA fragments extracted and purified (see 2.1.12). For cloning purpose, calf intestinal alkaline phosphatase (CIP) was added to the vector digestion mixture at the end of usual restriction reaction and incubated for additional 30 min at 37 °C. By catalyzing the release of 5' and 3' phosphate groups of the DNA digested fragments, the enzyme CIP was particularly useful to reduce self ligation of the vector DNA when single restriction sites were used.

2.2.11 Gel extraction

QIAquick gel extraction kit was used for the gel extraction and DNA fragments purification. After the restriction enzyme digestion, the products were separated by DNA electrophoresis, and the concentration of gel was selected on the size of target fragments. Generally, 1% agarose gel was used. The target DNA fragment was cut out from the gel under the UV light and placed into pre-weighed EP tube. To resolve the gel and release the DNA fragment, the QG buffer from the

QIAquick kit was added in a ratio of 3:1 (volume:weight) to the excised agarose gel containing the target fragment. The mixture was placed in the 50-60°C water bath and incubated for 10 min with vortexing every 2 or 3 min to melt the agarose to facilitate the release of DNA. When the gel was thoroughly melted, 100 µl isopropanol was added and mixed, followed by applying to the QIAquick spin column. A centrifugation at 13,000 rpm for 1 min allowed the DNA binding to the silica membrane while the QE buffer it was removed. 500 µl buffer QG was added following centrifugation for 1 min at 13,000 rpm to wash the bind DNA and remove the residual of agarose and ethidium bromide (EB). After removing the flow through, 750 µl washing buffer PE was added to the column and incubated for 3 min, followed by centrifugation for 1 min. An additional centrifugation for 1 min was applied to remove any residual washing buffer. Then the column with binding DNA was transferred to a clean 1.5 mL EP tube and proper volume (according to the estimated quantity of target DNA and the concentration needed) of elution buffer (EB) (10mM Tris-Cl, pH 8.5) or distilled water was added to the center of the column membrane. At then end of 3 min incubation, the EB buffer containing the target DNA fragment was collected by centrifugation for 1 min at 13,000 rpm.

2.3 Cell culture and transfection

2.3.1 Freezing, storage and recovery of cells

Cells were allowed to grow to 80-90% confluency in T75 tissue culture

flask (Corning, US). The culture medium was removed and cells were washed with pH7.2 $1 \times$ PBS (Gibco, USA) twice. Cells were then trypsinized with $2 \times$ Trypsine/EDTA in 37°C for several minutes,. When the cells were detached from the flask, the trypsinization was stopped by adding MEM with 10% FBS and cells were collected after centrifugation at 1,000rpm for 5min at room temperature (RT). The supernatant was discarded and the cell pellet was suspended in 3 mL cell freezing medium with 10% DMSO, 20% FBS even distributed in MEM. 1 mL of the suspended cell medium was then aliquoted into 2 mL sterile plastic ampoule with screw cap. The ampoule was placed in -20°C for 2 hours and followed by -80°C freezer overnight to allow gradual temperature decrease. The frozen cells were then rapidly transferred into liquid nitrogen tank for long term storage.

When taken out from the liquid nitrogen, cells were thawed by quickly and gently swirling the stored ampoule in 37°C water bath in less than 2min. The cell suspension was removed from the ampoule to sterile 15 mL tube, by adding 9 mL fresh MEM with 10% FBS. The medium with DMSO was removed by centrifugation at 1,000 /min for 5min at RT. The removal of DMSO was to minimize detrimental effect on cell growth. Cell pellet was suspended in 10 mL pre-warmed fresh MEM with 10% FBS and 1% anti-mycotic in T75 cell flask. The cells were maintained in 37°C and 5% CO_2 cell culture incubator. Cells freshly recovered were allowed to passage for 2 cycles before experiments to ensure a normal cell growth.

2.3.2 Cell culture and transfection with HBV genome

Three cell types including: 1. HepG2 cells transfected with empty pcDNA3.1 vector (pcDNA3.1 cells); 2. HepG2 cells transfected with a replicative wild type HBV genome cloned in pcDNA3.1 (wtHBV cells); 3. HepG2 cells transfected with a replicative mutant HBV genome cloned in pcDNA3.1 (mHBV cells) have been employed for our current investigation. The replicative HBV genome was constructed by cloning a linear form of viral genome into mammalian expression vector pcDNA3.1. Replicative genome consisting of $\sim 1.3\times$ HBV genome in length with terminal redundancy was constructed to reflect the *in vivo* environment, whereby the pre-genome RNA is the template for HBV replication [141].

HepG2 cells (ATCC, USA) were maintained in Gibco Dulbecco's minimal essential medium (MEM) (Invitrogen Inc., USA), complemented with 10% fetal bovine serum (Invitrogen Inc., USA) and 1% anti-mycotic (Invitrogen Inc., USA) under 37 °C and 5% CO₂. To maintain their viability, the cells were passaged by trypsinization with 2×Trypsin-EDTA/PBS at 37 °C and pH 7.2 (Invitrogen Inc., USA) every 3~4 days.

Transfection of HepG2 cells was carried out with Effectene transfection reagent (QIAGEN, USA) according to manufacturer's instructions. In brief, 6×10^5

cells were seeded and cultured on a 60 mm polystyrene dish (Nunc Inc., USA) under 37 °C and 5% CO₂ for 24 h. After reaching 40~50% confluency, the adherent cells were transfected with 1 µg of replicative wild type or mutant HBV genome (rHBV; genotype B) or empty pcDNA3.1. In detail, the plasmid constructs were mixed with 8 µl of enhancer, added up to 150 µl with TE buffer, cultured at room temperature for 5 mins, supplemented with 25 µl of Effectene transfection reagent and finally cultured at room temperature for 10 mins. After 3~4 h of transfection, the medium was removed. The cells were rinsed twice with 1×PBS before fresh medium was added. Transfected cells were maintained at 37 °C and 5% CO₂ for 48 h to allow HBV replication. Transfected cells were harvested by trypsinization, pelleted and re-suspended in fresh medium before carrying other measurements.

In the cell migration tracking assay, 1 µg of plasmid DNA encoding green fluorescence protein (pEGFP) was co-transfected with the three plasmids as mentioned above. With the expression of GFP, the successfully transfected cells produce strong fluorescence signal against the background for accurate detection on a fluorescence microscope in order to make the cell tracking feasible. Before cell seeding, the laminin-coated PIPAAm glass coverslips were maintained above LCST (at 37 °C) in order to keep the surface hydrophobic. HepG2 cells were then plated onto PIPAAm surface at 37 °C and returned to the CO₂ incubator in order to allow effective cell attachment. After 4 hours of seeding on the surface, the samples are immediately transferred to an online CO₂ incubator attached to our

microscope stage at 22 °C under a humidified atmosphere of 5% CO₂ for further biophysical measurements.

2.4 RNA extraction, quantitation and RT-PCR

2.4.1 RNA extraction

Total RNAs were extracted from HepG2 cells transfected by various plasmids by using the RNeasy Mini kit (QIAGEN, USA) according to the manufacturer's instructions. Briefly, cells were pelleted by trypsinization and resuspended in 350 or 600 µl (>5×10⁶ cells) of Buffer RLT (supplied by the kit) followed by homogenization for 30 sec by vortexing. An equal volume of 70% analytical grade ethanol was then added, and the mixture was applied to the spin columns (within 700 µl) and centrifuged at 13,200rpm for 30 sec. The columns were then washed with 700 µl of Buffer RW1 and centrifuged for 30 sec. After two additional washes with 500 µl Buffer RPE, additional centrifugation was applied to remove residual buffer RPE. The total RNA bound on the membrane of the column was eluted with 50 µl RNase-free water or DEPC treated water. Total RNA was quantified and stored at -80°C for long term storage.

2.4.2 RNA quantitation

The concentration and quality of RNA were measured by the absorbance reader at OD₂₆₀ and the absorbance ratio of OD₂₆₀ and OD₂₈₀. Extracted total RNA

was diluted 100 to 200× to perform the quantification. Yield was calculated by multiplication of by OD₂₆₀ 40 µg/mL and a further multiplication by the dilution factor. Quality and Purity of the RNA were calculated by division of OD₂₆₀ over OD₂₈₀.

2.4.3 Reverse transcript-polymerase chain reaction

The expression of HBV genome was verified by reverse transcript-polymerase chain reaction (RT-PCR) of *HBcAg* and *HBsAg* gene. After 48 hours of transfection, all three kinds of cells were separately collected and the total of mRNA was extracted with RNeasy Mini Kit (QIAGEN) according to the manufacturer's protocol. 1 µg of each type of total mRNA was used for amplification by using One Step RT-PCR Kit (QIAGEN). Primer sequences specific for *HBcAg*, *HBsAg* and β -*actin* gene in RT-PCR were shown in Table 2.2.

Table 2.2 Primer sequences specific for *HBcAg*, *HBsAg* and β -*actin* gene in RT-PCR.

<i>HBcAg</i> forward: 5'-ATCTCCTAGACACCGCCTCA-3'
<i>HBcAg</i> anti-sense: 5'-TTCCAAATTATTACCCACCC-3'
<i>HBsAg</i> sense: 5'-TCACCATATTCTTGGGAACAA-3'
<i>HBsAg</i> anti-sense: 5'-GTTTTGTTAGGGTTTAAATG-3'
β - <i>actin</i> sense: 5'-CTTAGTTGCGTTACACCCTTTC-3'
β - <i>actin</i> anti-sense: 5'-ACCTTCACCGTTCCAGTTTT-3'

The RT-PCR protocol for *HBcAg* and *HBsAg* gene are briefly described as follows: reverse transcription (RT) at 50 °C for 1 h; initial denaturation at 95 °C for 5 min; 25 cycles of PCR (30 s of denaturation at 95 °C, 30 s of annealing at 60 °C and 1

min of extension at 72 °C); final extension at 72 °C for 10 min. Upon the end of RT-PCR, 5 µl of the DNA products were loaded in 1.0% agarose gel within an electrophoresis tank for 30 min at 120 V. DNA was stained with ethidium bromide (Sigma, Singapore), visualized under UV light and imaged by ChemDot (Bio-Rad, USA).

2.5 IMX analysis

IMX system from Abbott Laboratories was used for qualitative and semi-quantitative analysis of HBV replication. In this system, the detection of HBsAg in culture medium was measured by the use of Microparticle Enzyme Immunoassay (MEIA) technology. This method has been widely used in clinical diagnosis for chronic HBV carriers.

The assay functions on the specific interaction of kit provided mouse monoclonal anti-HBs coated microparticles with HBsAg. The anti-HBs were biotinylated and when added into the antibody-antigen mixture, an antibody-antigen-antibody sandwich complex was formed. The complexes were then bound to the glass fiber matrix and followed by reaction with anti-biotin (Alkaline phosphatase conjugated). The unbound materials were washed away, and the substrate 4-Methylumbelliferyl phosphate was then added and the measurement was taken by the IMX MEIA optical assembly. In our experiments, cell transfected with plasmids, carrying HBV replicative genome was incubated for 48 hours at normal

cell culture condition to allow HBV replication and HBsAg secretion. The culture medium containing the secreted HBsAg was collected by centrifugation at 2,000 rpm for 10 minutes to remove the cell debris. Five drops (around 200 μ l) of culture medium was added to the proper carousel. Separately, the positive and negative controls were also added to different carousels. Detection results were reflected numerically. The waste tank was cleaned after each single use.

2.6 Cell culture and treatment with EGCG

HepG2 were from ATCC and cultured and passaged in Dulbecco's Minimal Essential Medium (DMEM) plus 10% fetal bovine serum (FBS) (Gibco, USA), 1% anti-mycotic (penicillin, streptomycin and amphotericin) (Invitrogen, USA) at 37°C 5% CO₂. HepG2.2.15 was cultured with DMEM (Gibco, USA), plus 10% FBS, 1% P+S (penicillin+streptomycin), 150 mg/ml of G418 (Geneticin) (Gibco, Invitrogen), 1 mM sodium pyruvate, 2 mM glutamine and 0.1 mM MEM NEAA under 37 °C 5% CO₂. The cells were passaged every 2~3 days until it reached approximately 80-90% confluency. For the cell passage, the cells were first washed by PBS (Gibco, USA), and treated with trypsin (Gibco, USA) for several minutes (depending on the cell type, 3-5 minutes usually). The cells were observed under inverted microscopy. The trypsinization was halted by adding culture medium when the cells turned round and were separating from each other and the cells were gently pipetted to disperse and detach from the surface and one to another. The suspended cells were centrifuged at less than 200g for 5 minutes

before adding fresh medium and aliquoted into flask in a proper concentration, usually 3 to 5 times dilution.

After 48 h of incubation, different concentrations of EGCG-treated HepG2 cells or HepG2.2.15 cells were harvested at a concentration of 10^5 /ml in fresh serum-free MEM medium. One ml of cells were seeded on each modified coverslip pasted onto a hollow cell culture dish. Then a corresponding concentration of EGCG was added for further biophysical measurements.

2.7 MTT Assay

Cytotoxicity was determined by the MTT reduction assay [142, 143]. The HepG2 cells were plated at 10^5 cells/ml. After 24 h of normal cell culture, the medium was removed and replaced with serum-free MEM medium supplemented with 2, 5, 10, 20 or 50 μ g/ml of EGCG (Sigma-Aldrich Inc., USA). After 24 h of incubation with EGCG, the MEM medium was discarded and cells were incubated at 37 °C for 3 h with 5 mg/ml MTT dissolved in PBS. Afterwards, MTT was removed and the formazan salts were solubilized in 100 μ l DMSO. Plates were read at 570 nm against a 660 nm reference wavelength on a microplate reader (Benchmark Plus). The cell viability was calculated as a percentage of the corresponding control value.

2.8 Confocal-reflection interference contrast microscopy (C-RICM)

The C-RICM system is based on a laser scanning confocal microscope (Pascal 5, Carl Zeiss, Germany) which is integrated with an online CO₂ incubator (Carl Zeiss, Germany). The illumination source is an argon ion laser with a maximum power of 1 mW and excitation wavelength of 488 nm. A 20× objective (NA: 1.25) was used in this study.

Different groups of HepG2 cells were cultured on laminin coated PIPAAm surface for 48 hours at 37 °C and 5% CO₂ atmosphere, and then refreshed with fresh medium at 37 °C. The sample was then transferred to an online CO₂ incubator attached on the microscope stage at 37 °C. The online incubator has the capability of rapid cooling (PE94, Linkam, UK). After 4 hours of incubation at 37 °C, the temperature was ramped down to 22 °C within 2 min in a 5% CO₂ atmosphere. In order to monitor the adhesion/deadhesion kinetics of HepG2 cells from laminin-coated PIPAAm-grafted coverslips below LCST, a series of C-RICM and phase contrast images on a selected region of the sample was taken against time. At each time point, fifty or more cells on at least three identical samples were imaged. By drawing a region of interest with a PC mouse using the ZSM 5 software (Carl Zeiss, Germany), the adhesion contact area and projected area of each cell is measured from the dark region (representing strong adhesion contact) and cell periphery in C-RICM image and phase contrast image, respectively. From the adhesion contact area and projected area, the average radius of adhesion

contact zone (a) and projected radius (R) of each cell were determined. Furthermore, the normalized adhesion contact area is defined as the ratio of adhesion contact area at any time point during cell deadhesion (A) and adhesion contact area before the temperature reduction at 37 °C (A_0). Then the degree of deformation of each cell which represents the cell geometry is simply defined as the ratio a/R .

2.9 Analysis of contact kinetics

The details of the contact mechanics model of adherent cells have been previously described in detail [144]. In brief, the equilibrated geometry of a cell adhering to a non-deformable substrate is modeled as a truncated sphere with a mid-plane radius R . The degree of deformation, $\sin\theta=(a/R)=\alpha$, is an experimentally measurable parameter, where R and a are measured by C-RICM and phase contrast microscopy, respectively. The cell wall is assumed to be under a uniform equi-biaxial stress, $\sigma=C\varepsilon$. C is the stiffness and calculated with the inputs of the elastic modulus E , membrane thickness h , Poisson's ratio ν and degree of deformation, respectively. The average biaxial strain, ε , is directly calculated from R and a as follows:

$$\varepsilon = \frac{1}{2} \left[\frac{2 + 2(1 - \alpha^2)^{1/2}}{4/R^2 - \alpha^2} - 1 \right] \quad (1)$$

In the absence of external force, the cell spontaneously adjusts its distance towards the substrate until equilibrium is achieved. It has been shown previously

that the adhesion energy, W , is as follows:

$$W = (1 - \cos\theta)C\varepsilon + C\varepsilon^2 \quad (2)$$

By taking the square root of the ratio of projected area (from phase contrast microscope) and adhesion contact area (from C-RICM), W can be found by Eqs. (1) and (2). For the case of an elliptical cell shape, a/R represents the ratio of the root mean square (RMS) radius for adhesion contact area($\sqrt{a \times b}$) and the RMS radius of the projected area($\sqrt{R_1 \times R_2}$), where $a(R_1)$ and $b(R_2)$ are the semi-major and semi-minor axis of the elliptical adhesion contact (projected shape), respectively. Thus a/R of an ellipse as mentioned above is useful for comparing the size of adhesion contact with that of the projected area. Despite the presence of an elliptical cell shape during the early stage of cell deadhesion, our approach to quantifying the extent of adhesion contact formation against the projected cell area as mentioned above remains valid. E , h and ν of HepG2 cell are respectively taken as 2000 N/m², 20 μ m and 0.5 according to the previous experimental results obtained from micropipette aspiration and AFM indentation [145].

2.10 Cell migration tracking assay

For the group of HepG2 cells transfected with HBV genome, after 48 hours of transfection, pcDNA3.1, wtHBV or mHBV cells co-transfected with pEGFP were harvested and transferred to an online CO₂ incubator which is integrated to a microscope stage with temperature controller. Cells were allowed to attach and

spread on laminin-coated PIPAAm-grafted glass coverslip for 4 hours of culture under 37 °C and 5% CO₂. Cell tracking of each type of GFP expressing HepG2 cells on laminin-coated PIPAAm-grafted coverslips for a duration of 4 hours at 22 °C and 5% CO₂ atmosphere was performed with time-lapse fluorescence microscopy (Olympus Inc.). The object tracking application of Meta Morph 7.0 software was then utilized for image analysis. The fluorescence images were automatically taken every 10 minutes within the 4 hours of 22 °C incubation. HepG2 cells expressing GFP were specifically recognized as fluorescence objects by Meta Morph 7.0 with their characteristic pixel appearance and distribution. Guided by the labels, we could track the pathway of each HepG2 cell from one frame to another. The cell displacement from its origin was recorded and measured in every 10 minutes, from which the root mean-square speed was calculated. Statistical data came from at least 50 cells of each type of cells.

For the group of HepG2 cells treated with EGCG, after 48 hours of treatment with EGCG, different HepG2 cells were harvested and transferred to an online CO₂ incubator which is integrated to a microscope stage, which is equipped with temperature controller. Cells were allowed to attach and spread on laminin-coated glass coverslip for 4 hours of culture under 37 °C and 5% CO₂. Cell migration tracking of each type of HepG2 cells on laminin-coated coverslips for a duration of 16 hours at 37 °C and 5% CO₂ atmosphere was performed with time-lapse fluorescence microscopy (Olympus, Japan). The object tracking

application of Meta Morph 7.0 software was then utilized for image analysis. The fluorescent images were automatically taken after every 20 minutes within the 16 hours of 37 °C incubation. HepG2 cells were specifically recognized by Meta Morph 7.0 with their characteristic pixel appearance and distribution. Guided by the labels, we could track the pathway of each HepG2 cell from one frame to another. The cell migration distance was recorded and measured in every 20 minutes, from which the average cell migration velocity was calculated. Statistical data came from at least 50 cells of each kind of samples.

2.11 Analysis of cell migration data

The persistent random walk model [146] was employed to characterize cell migration. The mean square displacement $\langle d(t)^2 \rangle$ at each time point t was calculated from the following formula:

$$\langle d(t)^2 \rangle = \frac{1}{M} \sum_{i=1}^M d_i(t)^2$$

Where M is the total number of cells tracked by the microscope and $d_i(t)^2$ is the displacement of each cell from during the respective time interval ($t=i\Delta t$). Then the values of $\langle d(t)^2 \rangle$ were plotted against time t and fitted by the persistent random walk equation as follows [146]:

$$\langle d(t)^2 \rangle = 2S^2 [Pt - P^2(1 - \exp(-\frac{t}{P}))]$$

where t is time, S is the root mean square speed of the cell population and P is the persistence of cell movement. The persistence is a measure of the time interval

between significant changes in the direction of cell movement. For long time $t > P$, the random walk equation ($\langle d(t)^2 \rangle$ vs. t) can be simplified to:

$$\langle d(t)^2 \rangle = 2S^2Pt - 2S^2P^2$$

where S and P were calculated from the slope ($2S^2P$) and the y-intercept ($-2S^2P^2$) of the plot.

2.12 Immunofluorescence staining

Two batches of cells at either 0 min or 30 min after adhesion or deadhesion at 22 °C from laminin-coated PIPAAm-grafted coverslips were stained with FITC-phalloidin (Sigma-Aldrich Inc., USA) and DAPI (Invitrogen Inc., USA) to label the actin and nucleolus, respectively. In brief, HepG2 cells were washed with 1× PBS and fixed with 3.7% formaldehyde in 1× PBS at room temperature for 10 min. After permeabilization with 0.2% Triton X-100 in 1× PBS for 10 min at room temperature, the cells were washed with 1× PBS and blocked with 10% FBS in 0.1% Triton X-100 for another 10 min. Then the cells were incubated with FITC-phalloidin (against actin) and DAPI (against nucleolus) for 1 h. Finally, the cells were washed twice with 0.1% Triton X-100 for 15 min. Fluorescence images of HepG2 cells stained with FITC-phalloidin and DAPI were acquired by a fluorescence microscope (Olympus, Japan) fitted with a 40× objective and an filtered light as the excitation source (488 nm and 300 nm) at a resolution of 4096×4096 pixels. Then the fluorescence images under two different excitation source were overlayed for further analysis.

2.13 LC-MS/MS Analysis on protein Profile

2.13.1 Total proteins extraction, protein digestion, and labeling with iTRAQ reagent for LC-MS/MS

The protocol was on the basis of our published work and most procedures were similar or identical [137]. Two days after transfection or treatment, cells were harvested and lysed in 250~400 μ l (depending on the cell concentration) of 8 M urea, 4% CHAPS (w/v), and 0.05% SDS (w/v) on ice for 30 min with regular vortexing. The mixture was centrifuged at 15, 000g for 60 min at 4 °C. After the supernatant was removed, the concentration of protein was measured with 2-D Quant Kit (GE Healthcare) or Bradford protein quantification assay. A standard curve was made using BSA as a control. Specifically, a total of maximum 100 μ g of each protein sample was precipitated by adding 4 volume of pre-cold acetone at -20 °C for 2 h. Then the precipitated proteins were pelleted by centrifugation at 15,000 rpm for 10 min. Before labeling with iTRAQ reagents, the proteins were dried and dissolved in 20 μ L dissolution buffer (0.5M triethylammonium bicarbonate, pH8.5 and 1.5 μ L 2% SDS), and denatured, and reduced by 2 μ L 50mM reduction buffer (TCEP-HCl, tris (2-carboxy-ethyl) phosphine) followed by incubation for 1 hour at 60°C. Afterwards, cysteines were alkylated by adding 1 μ L cysteine blocking buffer (200mM methyl-methanthiosulfonate (MMTs)) as described in the provided protocol in iTRAQ kit (Applied Biosystems, MA, USA).

Sample being analyzed was then digested with 20 μL of 0.25 $\mu\text{g}/\mu\text{L}$ sequence grade modified trypsin (Promega, USA) solution at 37 °C for 12~16 h and the iTRAQ tags were used to label them respectively as the following two batches (iTRAQ 114, 116, 117): HepG2 cells transfected with empty pcDNA3.1 vector = iTRAQ 114; HepG2 cells transfected with wildtype HBV genome = iTRAQ 116; HepG2 cells transfected with mutant HBV genome = iTRAQ 117; normal HepG2 cells vector = iTRAQ 114; HepG2 cells treated with 5 $\mu\text{g}/\text{ml}$ of EGCG = iTRAQ 116; HepG2 treated with 7.5 $\mu\text{g}/\text{ml}$ of EGCG = iTRAQ 117. The labeled samples were then combined for further analysis. To make sure that there was no interference between sample preparation and digestion/labeling, the bovine serum albumin (BSA) standard solution (Pierce) was used separately in the acetone precipitation, followed by enzymatic digestion with trypsin and labeling with the iTRAQ reagents as described above. These differentially labeled digests were mixed at a ratio of 1:2:1.5:2 and analyzed by LC-MS/MS [137].

2.13.2 Online LC-MS/MS Analysis

Peptides were analysed by Agilent 1200 nanoflow LC system (Agilent Technologies, US) interfaced with a 6530 Accurate-Mass Q-TOF LC/MS system (Agilent). A total 60 μL of the combined peptide mixture was injected into the the G4240-62002 HPLC chip system consisted of Zorbax 300SB-C18 enrichment column (4 mm 40 nl, 5 μm) and Zorbax 300SB-C18 separation column (150 mm $\times$

75 μm , 5 μm) and. Peptides were pressure loaded by a Capillary pump and a Nano pump. Peptides were eluted from the Capillary pump at a flow rate of 4 $\mu\text{l}/\text{min}$ to the enrichment pool and from the Nano pump at a flow rate of 300 nl/min to the mass spectrometer through an integrated nanospray emitter tip. Needle voltage was set to 1750 V. The mobile phase in Capillary pump used for a constant elution consisted of (A) 0.1% formic acid and (B) 10% acetonitrile, whereas the mobile phase in Nano pump used for a linear gradient elution of 50–90% B in 45 min followed by 90% B in 5 min and 90-10% in 10 min consisted of (A) 0.1% formic acid and (B) 90% acetonitrile. 15 runs (4 μl per run) were performed altogether for one injection.

Both MS and MS/MS spectra were acquired with the instrument operating in the data-dependent mode of one MS scan followed by three MS/MS scans. For MS/MS analysis, survey scans were acquired from m/z 300-2000 with up to two precursors selected for MS/MS from m/z 100-2000 using dynamic exclusion, and the rolling collision energy with a slope of 3.9 V/100 Da and a offset of 2.9 V was employed to promote fragmentation as described [137]. Ion signals above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode for generating collision-induced dissociation (CID) spectra.

2.13.3 Data Analysis and Interpretation

Peptide and protein identifications and relative abundance quantitation

were performed using Spectrum Mill software packages (Agilent). Each MS/MS spectrum was searched the specie of *homo sapiens* against the SwissProt protein database. A Spectrum Mill protein probability score of at least 7, a peptide probability score of at least 4 and a %SPI of at least 10 were used to filter the data, which gives the confidence value of more than 99%, followed by manual validation. All protein identifications made on single unique peptide were discarded. The searches were run using the following parameters: fixed modification of methylmethanethiosulfatelabeled cysteine; fixed iTRAQ modification of free amine in the N-terminus and lysine; variable iTRAQ modifications of tyrosine; allowing serine and threonine residues undergoing side reaction with the iTRAQ reagent.

Quantitative data analysis was performed using the Spectrum Mill software (Agilent). The mass tolerance settings was 20 ppm for precursor and 50 ppm for product. Spectrum Mill pooled data from all the series of runs in one experiment. All identifications were manually inspected to minimize machine-related errors. Relative quantification of proteins in the case of iTRAQ was performed on the MS/MS scans and was the ratio of the areas under the peaks at 114, 116, and 117 Da which were the masses of the tags that correspond to the iTRAQ reagents. The relative amount of a peptide in each sample was calculated by dividing the peak areas observed at 116.1, and 117.1 m/z by that observed at 114.1 m/z. The calculated peak area ratios were corrected for overlapping isotopic contributions,

and were used to estimate the relative abundances of a particular peptide. For proteins with two or more qualified peptide matches, two average peak area ratios (designated as 116/114 and 117/114) were calculated using the peak area ratios of the peptides originating from the same protein. The Distinct Summed MS/MS Search Score is Spectrum Mill's measurement of protein identification confidence taking into account all peptide evidence for a protein, excluding any evidence that is better explained by a higher ranking protein. AA% coverage is calculated by dividing the number of amino acids observed by the protein amino acid length. The candidate proteins were carefully examined in the Protein ID of ProteinPilot software. The peptides without any modification of free amine in the Nterminus or without iTRAQ modification of free amine in the lysine were excluded from calculation of the protein ratios. To account for small differences in protein loading, all protein ratios have been normalized using the overall ratios for all proteins in the sample.

2.14 Statistical Analysis

Values were reported as mean $\pm$ standard error. The student *t*-test was used for comparisons between different groups of HepG2 cells. Statistical significance was accepted at $p < 0.05$.

Chapter 3 HBV induced coupling of deadhesion and migration of HepG2 cells on TRP surface

In this part of my thesis, the complex interplay between cell adhesion and deadhesion kinetics, HBV replication and cytoskeletal protein modulation is elucidated with the use of real-time biophysical measurements. It is shown that HBV replication during the initial stage of cell seeding on laminin coated PIPAAm grafted surface perturbs the temporal trends of degree of cell deformation and adhesion energy of the cells. Interestingly, the biological origin of our observed HBV induced responses is also implied by the characterization of interaction between vinnexin- β and HBx, via a newly identified SH3-binding motif [63, 147]. This part will focus on the influence of this motif on cell adhesion and deadhesion kinetics. It is an extension of previous work on SH3 binding domain in HBx[63, 147]. Different from previous study, the whole HBV genome, instead of HBx gene, was introduced into the cell to acquire a more comprehensive and realistic result. In addition, the influence of the other three proteins expressed by HBV genome is also eliminated. Moreover, PIPAAm-grafted surface, with its transient inverse temperature phenomenon, was introduced to accelerate the much slower spontaneous cell deadhesion and migration process, making the observation much easier and ensuring the viability of cells.

3.1 The validation of transfection

In order to model HBV infection in hepatocytes, HepG2 cells were transfected with either empty pcDNA3.1 vector, wild type HBV or mutant HBV genome. In this study, four proline-to-alanine mutations (P39, P42, P43 and P46) were introduced in the SH3-binding domain of mutant HBV genome. It has been previously shown that significant amount of HBV particles was produced by HepG2 cells through the transfection with HBV genome [148]. Moreover, pEGFP plasmid encoding green fluorescence protein (GFP) was co-transfected into HepG2 for evaluating the transfection efficiency. The co-expressions of HBV genome and green fluorescence proteins were first validated by the high fluorescence signal detected in wtHBV cells under fluorescence microscope (Figure 3.1).

Figure 3.1 The co-expression of HBV genome and GFP was validated as shown by the high fluorescence signal detected in wtHBV cells with fluorescence microscope. A green dot represents a cell successfully expressing GFP.

The scale-bar represents 100 μm .

3.2 The validation of HBV replication

Figure 3.2 showed the RT-PCR products of *HBcAg* and *HBsAg* gene segments from pcDNA3.1, wtHBV or mHBV cells after gel electrophoresis. The clear DNA bands with the correct molecular weights on the gel indicated that mRNA encoding HBcAg and HBsAg proteins were successfully expressed in wtHBV and mHBV cells. As a negative control, pcDNA3.1 cells failed to express the mRNAs which encoded HBcAg and HBsAg proteins. Conventionally, β -actin was used as an internal control of RT-PCR. The significantly higher mRNA expressions of HBcAg/ HBsAg genes in wtHBV and mHBV cells compared to that of pcDNA3.1 cells was further supported by the slightly higher expression level of β -actin in pcDNA3.1 cells. The result as mentioned above supported that the specific expressions of HBcAg and HBsAg in HepG2 cells were achieved by the HBV replication system.

Figure 3.2 The electrophoresis agarose gel image of RT-PCR products of HBcAg and HBsAg gene segments generated from pcDNA3.1, wtHBV or mHBV cells.

3.3 The deadhesion kinetics of HepG2 under HBV replication

It is interesting to apply PIPAAm system for probing the biophysical responses of HepG2 cells under the influence of HBV replication. Figure 3.3 showed a series of phase contrast images (A, C, E) and C-RICM images (B, D, F) of a typical pcDNA3.1, wtHBV and mHBV cell seeding on laminin-coated PIPAAm surface against time after temperature reduction at 22 °C. As shown by the phase contrast images, all three types of cells initially demonstrated the typical round shape of HepG2 cells. From C-RICM image series, the adhesion contact area of the wtHBV cell was reduced from 1600 to 600 μm^2 (by around 63 %) from 0 to 75 min (Figure 3.3 D). The adhesion contact recession between cell and substrate was triggered by the acute increase of hydrophilicity of PIPAAm surface below LCST. In contrast, the adhesion of pcDNA3.1 or mHBV cells remained on PIPAAm below LCST was stronger than that of wtHBV cell. For instance, the adhesion contact area of the pcDNA3.1 and mHBV cell was reduced by 44 % and 46 %, respectively, during the initial 75 min of incubation at 22 °C (Figure 3.3 A and C). The result indicated that transfection of HepG2 cells with wild type HBV caused the cells to be more susceptible to thermally triggered deadhesion from the PIPAAm system.

Figure 3.3 A series of phase contrast (A, C, E) and C-RICM images(B, D, F) of a typical pcDNA3.1 cell (A, B), wtHBV cell (C, D) and mHBV cells (E, F) on laminin-coated PIPAAm-grafted glass which is taken at different times from 10 to 120 min after temperature reduction from 37 to 22 °C under 5% CO₂ atmosphere. The scale-bars represent 20 μm.

The result as mentioned above indicated that the mutations in the proline-rich region of *HBx* of HBV genome (by comparing mHBV with wtHBV cells) restored slower deadhesion of normal HepG2 cells (pcDNA3.1). Therefore the role of HBx protein in the laminin-mediated responses of HepG2 cells was significant. Our results possibly suggest an important role of the proline-rich region of HBx in SH3 binding during cell deadhesion since the proline-to-alanine mutations abrogated the enhancement in adhesion contact recession.

(A)

(B)

(C)

Figure 3.4 (A) A/A_0 , (B) a/R and (C) averaged adhesion energy for a population of pcDNA3.1 cell (◆), wtHBV cell (●) and mHBV cell (▲) against time of 22 °C incubation on laminin-coated PIPAAm surface.

The error bar of each data point represented the standard error of 50 cells on at least three independent samples.

Cell deadhesion has been conveniently quantified by the use of normalized adhesion contact area (A/A_0). Figure 3.4 A showed A/A_0 for a population of pcDNA3.1 cells (◆), wtHBV cells (●) and mHBV cells (▲) against time on laminin coated PIPAAm surface at 22 °C. The error bar of each data point represented the standard error of 50 cells on at least three samples. The result generally indicated that A/A_0 was reduced against time for all three types of cell. Moreover, the rate of reduction of A/A_0 of wtHBV cells from 0 to 75 min was 44.4 % higher than that of pcDNA3.1 cells. After 2 hours, A/A_0 of wtHBV cells

was 25.6 % lower than that of pcDNA3.1 cells. Transfection of mutant HBV genome in HepG2 cells did not lead to notable change in the trend of A/A_0 compared with that of pcDNA3.1 cells. The result indicated that A/A_0 portrayed the effect of HBV replication on the adhesion contact recession of HepG2 cells.

The delay of deadhesion of wtHBV cells may be accompanied by other biophysical responses as well. Figure 3.4 B showed the degree of deformation (a/R) of a population of pcDNA3.1 cells (◆), wtHBV cells (●) and mHBV cells (▲) against time on laminin-coated PIPAAm surface at 22 °C. The error bar of each data point represented the standard error of 50 cells on at least three samples. The result indicated that a/R of wtHBV cells readily dropped from 1 to 0.78 during the initial 75 min of seeding. Moreover, the initial rate of reduction of a/R for wtHBV cells is 0.0029 min^{-1} . In contrast, the rate of reduction of a/R of both pcDNA3.1 and mHBV cells is 46.2 % and 45.5 % respectively, and is lower than that of wtHBV cell. When a/R approached 1, the ventral membrane of the cell formed almost complete contact with the underlying substrate. The reduction of a/R from 1 to 0 signified the drastic change of cell geometry from a flattened disc to perfect sphere. The difference in the trend of a/R between normal and HBV transfected cell as mentioned above demonstrated that HBV replication directly tuned the initial geometry transformation of HepG2 cells during the thermally triggered deadhesion. From 75 to 120 min, a/R of pcDNA3.1 or mHBV cells remained to around 5% higher than that of wtHBV HepG2 cells. After 120 min, a/R of all the

three types of cells dropped to around 0.76. Based on the results of A/A_0 and a/R , HepG2 cells were less susceptible to HBV replication during the later stage of thermal induced deadhesion on PIPAAm surface.

Figure 3.4 C showed the averaged adhesion energy of a population of pcDNA3.1 cells (◆), wtHBV cells (●) and mHBV cells (▲) against time on laminin coated PIPAAm surface at 22 °C. The error bar of each data point represented the standard error of 50 cells on at least three samples. The result indicated that adhesion energy of wtHBV cells was reduced by 40 % from 0 and 75 min on PIPAAm surface below the LCST of PIPAAm. On the other hand, the adhesion energy of both pcDNA3.1 and mHBV cells remained constant compared to that of wtHBV cells during the same period. The trend as mentioned above was related to the faster deadhesion and shape transformation in wtHBV cells.

The deadhesion of HepG2 cells was significantly accelerated through SH3 binding during HBV replication. Moreover, the enhancement as mentioned above was abolished by four proline-to-alanine mutations in the proline-rich region of SH3 binding domain of HBx. The faster decay of adhesion energy was likely caused by the cytoskeleton remodeling in response to HBV replication [149]. Our findings in HBV-replicating HepG2 cells herein were supported by the delay in adhesion contact formation [63] and reported interaction between key cytoskeleton proteins and HBV-associated proteins [149].

3.4 Cell migration profile under HBV replication

Generally, cell migration is driven by a highly orchestrated cascade of adhesion and deadhesion [150]. On the other hand, the quantitative correlation between cell deadhesion and migration of HBV transfected cells on PIPAAm surface remained to be elucidated. Figure 3.5 showed the overlay of fluorescence images (at 0 and 4 hours after temperature reduction) for pcDNA3.1, wtHBV and mHBV cells on laminin-coated PIPAAm-grafted surface at 22 °C and 5% CO₂. The result indicated that the overall net displacements of wtHBV cells during the time interval were significantly higher than those of mHBV or pcDNA3.1 cells as shown by the migration tracks (redlines in Figure 3.5). Moreover, pcDNA3.1 and mHBV cells seemed to oscillate continuously at the original positions rather than achieving a significant net displacement.

Figure 3.5 The overlay of fluorescence images (at time 0 and 4 hours) of the migration tracks (the redline) of pcDNA3.1, wtHBV and mHBV cells on laminin-coated PIPAAm-grafted surface during 4 hours of incubation at 22 °C and 5% CO₂. The scale-bar represents 100 μm.

3.5 Persistence random walk analysis of cell migration under HBV replication

Cell migration on biomaterial surface is best modeled by the persistent random walk model [146]. Figure 3.6 A showed the root mean-square displacement $\langle d(t)^2 \rangle$ against time for at least fifty pcDNA3.1, wtHBV and mHBV cells on laminin-coated PIPAAm surface. First, $\langle d(t)^2 \rangle$ of wtHBV cells is significantly higher than that of pcDNA3.1 or mHBV cells during 4 hours of seeding at 22 °C. After 4 hours, $\langle d(t)^2 \rangle$ of wtHBV cells reached 9981.47 μm^2 , which is two orders of magnitude higher than those of pcDNA3.1 cells (65.86 μm^2) and mHBV cells (410.20 μm^2). The results strongly suggested that HBV replication led to larger extent of cell movement during 4 hours interval through the proline-to-alanine mutations.

Figure 3.6 (A) the root mean-square displacement $\langle d(t)^2 \rangle$ against time t of at least 50 pcDNA3.1, wtHBV and mHBV cells on laminin-coated PIPAAm surface. (B) The persistence time (P) vs. root mean-square speed (S) of at least 50 pcDNA3.1, wtHBV and

mHBV cells on laminin-coated PIPAAm surface. The instantaneous speed of each cell is calculated from the slope and intercept of the trendline of $\langle d(t)^2 \rangle$ vs. t curve.

Figure 3.6 B showed the persistence time (P) and root mean-square speed (S) of at least 50 pcDNA3.1, wtHBV and mHBV cells on laminin-coated PIPAAm surface during four hour interval. The averaged instantaneous speed for each type of cells is calculated from the slope and intercept of the trendline of $\langle d(t)^2 \rangle$ vs. t curve. The persistence time of wtHBV cells (79.89 min) was significant lower than that of pcDNA3.1 cells (132.76 min) and mHBV cells (120.89 min). The result indicated that HBV replication promoted frequency in the change of migration directions of HepG2 cells. Additionally, the root mean-square speed of pcDNA3.1, mHBV cells and wtHBV cells was 0.0478, 0.1194 and 0.6274 $\mu\text{m min}^{-1}$, respectively. The difference as mentioned above between HBV transfected cells and normal cells was verified by paired Student's t-test ($p < 0.05$). Hence, HBV replication led to simultaneous changes in both cell persistence time and motility during the thermally triggered transformation of PIPAAm surface.

3.6 Cytoskeleton remodeling under HBV replication

Cytoskeleton remodeling plays a crucial role in various cellular activities, such as migration, mitosis, apoptosis, differentiation, proliferation, etc. To visualize the cytoskeleton alteration of HepG2 cells on PIPAAm surface,

immunostaining was applied to label actin for subsequent visualization under fluorescence microscopy. Figure 3.7 showed the immunofluorescence images of actin for pcDNA3.1 cell, wtHBV cell and mHBV cells after 0 and 30 mins of deadhesion at 22 °C on laminin-coated PIPAAm surface. At 0 min, actin microfilaments were abundantly found at the cell periphery of both pcDNA3.1 and mHBV cells. The result indicated that pcDNA3.1 and mHBV cells were activated by the laminin on PIPAAm surface through the formation of dense actin network. In contrast, wtHBV cell did not demonstrate the formation of dense network of actin microfilaments at 0 min. From 0 to 30 min, both pcDNA3.1 and mHBV cells demonstrated the transformation of the dense actin microfilament network to sparsely distributed actin filaments following the contraction of cell body during cell deadhesion. Interestingly, actin became highly concentrated at the periphery of wtHBV cells after deadhesion for 30 min. The result strongly indicated that the localization of actin at cell periphery may provide the necessary traction for HBV trransfected cell to pull its membrane away from the PIPAAm surface during cell deadhesion and migration as observed herein.

Figure 3.7 Immunofluorescence microscopy image for actin of pcDNA3.1, wtHBV and mHBV cells at 0 min and 30 min after cell seeding at 22 °C on laminin-coated PIPAAm surface.

The scale-bars represent 20 μm .

3.7 Discussion & Conclusion

Until now, cellular and molecular mechanisms underlying intrahepatic metastasis of HCC (Hepatocellular carcinoma) remain obscure. Tumor invasion could be induced by the deregulation or moderation of cell migration [151]. It is necessary for a cancerous cell to down-regulate adhesion and up-regulate deadhesion in order to migrate. Firstly, a cell extends its protrusions such as lamellipodia and filopodia at the front end and subsequently retracts its tail through a synergistic cascade of adhesion and deadhesion. Thus the correlation between cell migration parameters and deadhesion kinetics of HepG2 cells under the influence of HBV replication as reported herein suggested the possible role of HBV in triggering the metastasis of tumor cells. The result herein was supported by the fact that cell migration is driven by a series of adhesion and deadhesion events between cell and external substrate through the dynamic assembly/disassembly of focal adhesions [152].

In this study, we applied PIPAAm system for elucidating the effect of HBV replication on the intricate biophysical responses of HepG2 cells. First of all, the transfection of replicative HBV genome was applied as a model system of HBV infection. By introducing mutations in proline-rich region, the impact of SH3 binding domain on the thermally triggered deadhesion and migration of HepG2 cells was exploited. It was demonstrated that HBV replication directly moderated the cell deadhesion kinetics on PIPAAm surfaces. Furthermore, cell migration was shown to be enhanced by HBV replication. It was demonstrated that PIPAAm

system in combination with our biophysical approaches can be used as a highly quantitative tool for elucidating the highly intricate cellular responses under the influence of viral infection or other biological stimulations.

Chapter 4 The modulatory behavior of EGCG in adhesion/deadhesion and migration of HepG2 Cells

To date, most studies of EGCG focused on its impact on proliferation, apoptosis and modulation of multiple signaling pathways whereas our aiming at contact kinetics and cell motility provides a novel quantitative perspective. Moreover, many results regarding the modulatory roles of EGCG in cell migration were reported in various carcinoma models including lung cancer, breast cancer, prostate cancer and gastric cancer [109, 122-126]. The quantitative work based on hepatocellular carcinoma remained rare.

A human liver carcinoma cell line, HepG2, was employed in this study as a liver cancer cell culture model system. Since cell adhesion, deadhesion and their dynamic assembly, migration, are fundamental activities and essential mechanisms underlying cancer invasion and metastasis, we investigated the consequence of EGCG treatment on cell adhesive, deadhesive and migratory behavior in order to develop new approach to evaluating therapeutic effect of EGCG. Firstly, HepG2 cells were treated with different concentrations of EGCG. Then the adhesion contact kinetics and cell geometry of various types of HepG2 cells during both adhesion and thermally triggered deadhesion were probed with the application of the confocal reflection interference contrast microscopy (C-RICM). Additionally, the immunofluorescence microscopy was applied to investigate the structural

transformation of actin of HepG2 cells. Furthermore, the cell migration under EGCG treatment was explored with time-lapse fluorescence microscopy.

4.1 MTT assay under EGCG treatment

To determine the appropriate concentrations of EGCG used in our study, HepG2 cells were incubated with concentration gradient of EGCG and the cytotoxicity was measured by the MTT reduction assay. Fig. 4.1 showed the cell viability of HepG2 cells after exposure to concentration gradient of EGCG for 24 h. The cell viability exhibited a dose-dependent behavior against EGCG and is drastically decreased to 90% under higher than higher EGCG concentration of 10 $\mu\text{g/ml}$. The results further indicated that EGCG imposes very low cytotoxicity to HepG2 cells at the concentration lower than 10 $\mu\text{g/ml}$. Hence 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG were chosen for further biophysical measurements.

Figure 4.1 Cell viability of HepG2 cells after 24 h of exposure to gradient concentrations of EGCG.

The error bar of each data point represented the standard error of three independent samples.

4.2 Adhesion kinetics under EGCG treatment

Generally, cell adhesion is known to be associated with receptor-mediated signal transduction cascades, such as the receptor expressions, ligand affinity, focal adhesion formation and cytoskeleton remodeling, etc. [153, 154]. In this study, laminin was selected as model ECM protein coated on thermo-responsive polymer

surface to forge the specific interaction with cell surface receptors. The kinetics of adhesion contact formation of HepG2 cells in response to EGCG treatments was explored. After 48 h of incubation with 5 $\mu\text{g/ml}$ or 7.5 $\mu\text{g/ml}$ of EGCG, HepG2 cells were seeded on laminin-coated PIPAAm-grafted glass coverslip. Furthermore, HepG2 cells without any EGCG treatment were used as control. Fig. 4.2 showed a series of phase-contrast images and C-RICM images of a typical normal HepG2 cell (A, B), HepG2 cell incubated with 5 $\mu\text{g/ml}$ of EGCG (C, D) and 7.5 $\mu\text{g/ml}$ (E, F) of EGCG taken at different times from 10 to 120 min after seeding on laminin-coated PIPAAm-grafted glass coverslip at 37 °C under 5% CO₂ atmosphere.

Figure 4.2 A series of phase contrast (A, C, E) and C-RICM images(B, D, F) of a typical normal HepG2 cell (A, B), HepG2 cell incubated with 5 $\mu\text{g/ml}$ (C, D) and 7.5

$\mu\text{g/ml}$ (E,F) of EGCG taken at different times from 10 to 120 min after seeding on laminin-coated PIPAAm-grafted glass coverslip at 37 °C under 5% CO₂ atmosphere.

The scale-bars represent 20 μm .

The phase contrast image first showed that all three types of cells became firmly bound on the laminin-coated substrate immediately following cell seeding. Normal HepG2 cells (Fig. 4.2 A) didn't spread on the surface until 50 min of cell seeding as shown by the lack of lamellipodia formation at cell periphery. During the initial 20 min, HepG2 cells incubated with 5 $\mu\text{g/ml}$ (Fig. 4.2 C) and 7.5 $\mu\text{g/ml}$ (Fig. 4.2 E) of EGCG didn't display significant spreading on the laminin-coated surface as shown by the round geometry of cells (Fig. 4.2 C and 4.2 E). From 30 min to 40 min, EGCG-treated cells began to spread through the formations of lamellipodia at cell periphery. The result strongly indicated that EGCG treatment significantly accelerated cell spreading triggered by integrin-laminin interaction on PIPAAm surface.

Conventional light microscopy such as phase-contrast microscopy only reveals the cell morphology and shape on the substrate. In order to elucidate the exact mechanism of cell-substrate interaction during cell seeding on biomaterials or ECM coated substrates, it is necessary to measure the adhesion contact area where cell membrane got very close to the opposing surfaces. C-RICM probes adhesion contact area through the destructive interference between the reflected lights from both cell membrane and model surface [155]. C-RICM results showed

that HepG2 cells incubated with the concentrations of EGCG developed notable adhesion contact after 20 min of cell seeding (cluster-like structures on Fig. 4.2 D and F). Interestingly, the adhesion contact region of the normal HepG2 cell remained negligible within the initial 20 mins and did not exhibit significant growth until 60 min after cell seeding (Fig. 4.2 B). The result indicated that the rate of adhesion contact formation for HepG2 cells was enhanced by EGCG.

Fig. 4.3 A showed the normalized adhesion contact area of a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ of EGCG (◆) and HepG2 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG (▲) against time of cell seeding on laminin-coated PIPAAm surface at 37°C under 5% CO_2 atmosphere. The error bar of each data point represented the standard error of 50 cells on at least three independent samples. The result generally indicated that normalized contact area (A/A_0) was increased against time for all three types of cells due to the adhesion spreading at 37°C. Moreover, the rate of increase of A/A_0 of normal HepG2 cells from 0 to 50 min was 15% higher than that of HepG2 cells treated with EGCG.

The combination of phase contrast microscopy and C-RICM determines the general shape of adherent cells on model substrates. The degree of cell deformation (a/R) is a key biophysical parameter which quantitatively compares cell spreading and adhesion contact formation for cell on a planar substrate. In this study, a/R was used for characterizing the influence of EGCG treatment on the

geometry of seeded HepG2 cells. Fig. 4.3 B showed a/R of a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ of EGCG (◆) and HepG2 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG (▲) against time of cell seeding on laminin-coated PIPAAm surface at 37°C under 5% CO₂ atmosphere. Each error bar represented the standard deviation of the data from at least 50 cells on three substrates. Generally, a/R of the three kinds of HepG2 cells rapidly increased against time during the initial 10 min of cell seeding (Fig. 4.3 B). However, the extent of the increase of a/R for normal HepG2 cells is less than the other two types of EGCG treated cells during the initial 10 min of cell seeding. The initial cell deformation rate for HepG2 cells treated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG was 0.00475 and 0.00538 min^{-1} , respectively. In contrast, the initial cell deformation rate of normal HepG2 cells (0.00425 min^{-1}) was 10~20 % lower than that of HepG2 cells treated with EGCG. The results supported that the kinetics of cell deformation is directly modulated by EGCG treatment.

(A)

(B)

(C)

Figure 4.3 (A) A/A_{120} , (B) a/R and (C) averaged adhesion energy for a population of normal HepG2 cells (●), HepG2 cells incubated with 5 µg/ml (◆) and 7.5 µg/ml (▲) of EGCG against time of cell seeding on laminin-coated PIPAAm surface at 37 °C under 5% CO₂ atmosphere.

The error bar of each data point represented the standard error of 50 cells on at least three independent samples.

Cell adhesion to ECM or biomaterials is a highly intricate process [65] and has been probed by various biophysical techniques through the determination of adhesion energy [156, 157]. A recently developed contact mechanics model of adherent cell has been successfully used to determine the adhesion energy of HepG2 cells [156, 158]. In our previous study, this approach has been applied to

investigate the alteration of adhesion dynamics in response to HBV replication [159]. Fig. 4.3 C showed the average adhesion energy of a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ (◆) and HepG2 cells incubated with 7.5 $\mu\text{g/ml}$ (▲) of EGCG against time of cell seeding on laminin-coated PIPAAm surface at 37 °C under 5% CO₂ atmosphere. It was first indicated that HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG rapidly achieved a notable adhesion energy of $0.96 \times 10^{-7} \text{ J/m}^2$ and $1.00 \times 10^{-7} \text{ J/m}^2$ at 20 min after seeding on laminin-coated substrate. In contrast, the averaged adhesion energy of normal HepG2 cells was about $0.25 \times 10^{-15} \text{ J/m}^2$ lower than those of the EGCG treated cells during the initial period of 20 min. The result indicated that the increase of adhesion energy is significantly accelerated by EGCG in normal HepG2 cells. Moreover, adhesion energies of HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG took about 50 min to reach steady state level of around $1.64 \times 10^{-7} \text{ J/m}^2$. Similarly, the adhesion energy of normal HepG2 cells took longest time of about 75 min in reaching a steady state compared to the other two types of EGCG treated cells. At steady state, the adhesion energy of HepG2 cells treated with EGCG also reached around $1.64 \times 10^{-7} \text{ J/m}^2$ which was the same as that of normal cells. The result strongly indicates that EGCG modulated the kinetics of adhesion energy evolution during cell seeding.

4.3 Deadhesion kinetics under EGCG treatment

Adhesive strength between cell and opposing surface can be depicted by either the force during initial encounter between cell and surface or that required for detaching adhering cell from the surface. Thus we hypothesized that the deadhesion kinetics of adherent cells on ECM-coated TRP would also be affected by EGCG treatment as well. Fig. 4.4 showed a series of phase contrast images (A, C, E) and C-RICM images (B, D, F) for a typical normal HepG2 cell (A, B), HepG2 cell incubated with 5 $\mu\text{g}/\text{ml}$ of EGCG (C, D) and 7.5 $\mu\text{g}/\text{ml}$ of EGCG (E, F) on laminin coated PIPAAm-grafted glass taken at different times from 10 to 120 min after temperature reduction from 37 to 22 °C under 5% CO₂ atmosphere. As shown by the phase contrast images, all three types of cells demonstrated the typical round or polygon shape at the initial phase of temperature reduction. From the time series of C-RICM images, the adhesion contact area of normal HepG2 cell was largely reduced by more than half from 0 to 50 min after temperature reduction to 22 °C (Fig. 4.4 B). The loss of close contact between cell and substrate was triggered by the acute increase of hydrophilicity of PIPAAm surface below LCST. In contrast, HepG2 cell treated with 5 $\mu\text{g}/\text{ml}$ or 7.5 $\mu\text{g}/\text{ml}$ of EGCG remained to be strongly adherent on PIPAAm below LCST compared to normal cell. Moreover, the adhesion contact area of HepG2 cell treated with 5 $\mu\text{g}/\text{ml}$ and 7.5 $\mu\text{g}/\text{ml}$ of EGCG was much less diminished during the initial 50 min of incubation at 22 °C (Fig. 4.4 C). The result indicated that the interaction with EGCG enhanced the adhesion strength of HepG2 cells by making them less susceptible to thermally triggered deadhesion from the laminin-coated PIPAAm

surface.

Figure 4.4 A series of phase contrast (A, C, E) and C-RICM images(B, D, F) of a typical normal HepG2 cell (A, B), HepG2 cell incubated with 5 $\mu\text{g/ml}$ (C, D) and 7.5

$\mu\text{g/ml}$ (E,F) of EGCG on laminin coated PIPAAm-grafted glass taken at different times from 10 to 120 min after temperature reduction from 37 to 22 °C under 5% CO₂ atmosphere.

The scale-bars represent 20 μm .

The deadhesion of HepG2 under various EGCG treatments was conveniently quantified by normalized adhesion contact area. Fig. 4.5 A showed the normalized adhesion contact area of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ of EGCG (◆) and HepG2 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG (▲) on laminin-coated PIPAAm surface against time after temperature reduction from 37 to 22 °C under 5% CO₂ atmosphere. The error bar of each data point represented the standard error of 50 cells on at least three independent samples. The result generally indicated that normalized contact area (A/A_0) was reduced against time for all three types of cell due to the adhesion contact recession from the PIPAAm surface below LCST. Moreover, the rate of reduction of A/A_0 of normal HepG2 cells from 0 to 50 min was two times higher than that of HepG2 cells treated with EGCG. Towards 2 hours of 22 °C incubation, A/A_0 of normal HepG2 cells was 60~70 % lower than that of the two types of EGCG-treated cells. The result indicated that incubation with EGCG led to significant change in the trend of the kinetics of adhesion contact recession.

Normally, the delay of deadhesion after EGCG treatment might be

accompanied by other concurrent changes of biophysical parameters of adherent cells. Thus it was crucial to characterize the geometry transition of EGCG treated cells during the thermal induced deadhesion on laminin-coated PIPAAm-grafted surface with the use of a/R . Fig. 4.5 B showed a/R of a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ of EGCG (◆) and HepG2 cells incubated 7.5 $\mu\text{g/ml}$ of EGCG (▲) against time after temperature reduction from 37 to 22 °C under 5% CO₂ atmosphere. The error bar of each data point represented the standard error of 50 cells on at least three independent samples. First of all, the initial rate of reduction of a/R for normal HepG2 cells was 0.0053 min⁻¹, whereas the rate of reduction of a/R for HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG was 84.4 % and 87.5 % lower than that for normal cell, respectively, during the initial 30 min. Moreover, the initial rate of reduction of a/R for normal HepG2 cells was 0.0082 min⁻¹ during the initial 50 min. Meanwhile, the rate of reduction of a/R for HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG was 34.1 % and 51.2 % lower than that for normal cell, respectively. The result also indicated that a/R of normal HepG2 cells rapidly dropped to around 0.74 and 0.59 during the initial 30 and 50 min of seeding respectively. When a/R approached 1, the ventral membrane of the cell nearly formed complete contact with the underlying substrate. The reduction of a/R from the limit 1 to 0 denoted the drastic transformation of cell shape from more flattened disc to perfect sphere. The difference in the trend of a/R in the three types of cell as mentioned above demonstrated that EGCG directly tuned the mechanism of geometry

transformation of HepG2 cells during the initial stage of thermally triggered deadhesion. From 50 to 90 min, a/R of HepG2 cells treated with 5 $\mu\text{g}/\text{ml}$ and 7.5 $\mu\text{g}/\text{ml}$ of EGCG remained to be higher, respectively, than that of normal HepG2 cells on laminin-coated PIPAAm surface. After 120 min of seeding, a/R of all the three types of HepG2 cells dropped to around 0.45. Furthermore, throughout the 120 min of culture at 22 °C, average a/R of HepG2 cells treated with 7.5 $\mu\text{g}/\text{ml}$ of EGCG was 3~10% higher than cells treated with 5 $\mu\text{g}/\text{ml}$ of EGCG. The result demonstrated that EGCG interfered the thermal deadhesion on PIPAAm surface induced in a dose-dependent manner.

(A)

(B)

(C)

Figure 4.5 (A) A/A_0 , (B) a/R and (C) averaged adhesion energy for a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ (◆) and 7.5 $\mu\text{g/ml}$ (▲) of EGCG against time after temperature reduction from 37 to 22 °C under 5% CO_2 atmosphere. on laminin-coated PIPAAm surface.

The error bar of each data point represented the standard error of 50 cells on at least three independent samples.

The averaged adhesion energy of a population of normal HepG2 cells (●), HepG2 cells incubated with 5 $\mu\text{g/ml}$ of EGCG (◆) and HepG2 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG (▲) against time after temperature reduction from 37 to 22 °C under 5% CO_2 atmosphere was shown in Fig. 4.5 C. The error bar of each data point represented the standard error of 50 cells on at least three independent samples. The result indicated that adhesion energy of normal HepG2 cells was reduced by 57.3 % from 0 and 30 min of thermally induced deadhesion. On the other hand, the adhesion energy for HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG almost remained constant during the same period of time at 22 °C. The trend as mentioned above was related to the lower deadhesion rate and shape transformation but the biological factors behind the detected changes needed to be further elucidated.

Images taken from C-RICM and phase contrast microscopy showed how different concentration of EGCG will affect the adhesion/deadhesion rate and eventually the migration rate of the HepG2 cells. At a temperature that was

unfavourable to cell adhesion, the rate of adhesion/deadhesion of HepG2 cells treated with different concentration of EGCG (0 $\mu\text{g/ml}$, 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$) increased/decreased with increasing concentration of EGCG. Graphs for the normalized contact area, the degree of deformation and adhesion energy of the HepG2 cells for the duration of 2 hours illustrated that the presence of EGCG had an enhancing/inhibitory effect on cell adhesion/deadhesion. Furthermore, the effect was amplified when the concentration of EGCG is increased. As such, the results exhibited were in line with the claim that EGCG enhances cell adhesion and inhibits cell deadhesion [160]. The trend as mentioned above was related to the slower deadhesion and shape transformation but the fundamental driving force behind the change needed to be further elucidated.

4.4 Actin remodeling under EGCG treatment

It can be postulated that of the alteration on the kinetics of adhesion energy was driven by the change in mechanochemical responses on ECM-coated surface such as the cytoskeleton alteration in response to EGCG treatment. Our findings on the accelerated cell adhesion and decelerated cell deadhesion of EGCG-treated HepG2 cells were supported by the previously reported interaction between key cytoskeleton proteins and EGCG-associated proteins [122, 123, 160-162]. More importantly, our work provided new quantitative perspectives on the phenotypic responses of HepG2 cells induced by the interaction between EGCG-associated

proteins and cytoskeleton proteins.

Figure 4.6 Overlay immunofluorescence microscopy image for actin (green) and nucleus (blue) of normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG at 10 min and 50 min (A) after cell seeding at 37 °C and (B) after temperature reduction from 37 to 22 °C on laminin-coated PIPAAm surface.

The scale-bars represent 10 μm .

It was shown that the cells were adhering to the PIPAAm surface at 37 °C.

However, when the temperature was lowered to 22 °C, cell deadhesion was induced and the cells started to retract from the surface and became more spherical in shape as shown in the images taken. This further confirmed the validity of PIPAAm in inducing cell adhesion and cell deadhesion at different temperature [163].

Cell motility is driven by assembly and disassembly of actin filaments. Universally, cell migration on ECM-coated surface or biomaterial consists of a cycle of four steps: protrusion of the front edge, adhesion to the substrate, retraction of the rear edge and deadhesion from the substrate [164]. To verify the differential actin filaments remodeling during cell adhesion and deadhesion, double-immunostaining was employed to label the actin and nucleus for fluorescence microscopy. Fig. 4.6 A showed the overlay immunofluorescence microscopy image for actin (green) and nucleus (blue) of normal HepG2 cells, HepG2 cells incubated with 5 µg/ml and 7.5 µg/ml of EGCG at 10 min and 50 min after cell seeding at 37 °C on laminin-coated PIPAAm surface. At 10 min, actin filaments mainly concentrated at the periphery of all types of cells, indicating cells were ready to initiate the adhesion. At 50 min, protrusions were found at the leading border of normal HepG2 cell whereas considerable concentrated actin was still located at the inner outline of cell. However, both HepG2 cells treated with EGCG outshot a large amount of filopodia and clear branched actin filaments were spotted at the leading edge in a circumferential fashion. The results suggested

the faster assembly of actin filaments caused by the incubation, which was in line with the higher rate of increase of adhesion energy. Fig. 4.6 B showed the overlay immunofluorescence microscopy image for actin (green) and nucleus (blue) of normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG at 10 min and 50 min after temperature reduction from 37 to 22 $^{\circ}\text{C}$ on laminin-coated PIPAAm surface. At 10 min, actin distributed throughout all types of HepG2 cells except that some actin filaments appeared at the periphery and aligned along the direction of retraction. It suggested both thorough spreading and readiness to deadhere of all cells. After 50 min, normal HepG2 cells demonstrated strong retraction while concentrated stress fibres surrounded the outline. In contrast, HepG2 cells treated with EGCG was much more relaxed. Some sign of retraction, consisting of actin filaments, appeared around the cell border. The result indicated that much weaker disassembly took place in EGCG-treated HepG2 cells, which is consist with its lower decreasing rate of adhesion energy.

4.5 Cell migration profile under EGCG treatment

Cell migration is conventionally regarded as a continuous cascade of adhesion and deadhesion events [150]. Nevertheless, the possible quantitative correlation between cell adhesion, deadhesion and migration under EGCG treatment remained to be clarified. Fig. 4.7 showed the overlay phase-contrast images of the migration track (the redline) of normal HepG2 cells, HepG2 cells

incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm-grafted surface in 16 hours of incubation at 37 °C under 5% CO_2 atmosphere. All types of HepG2 cells displaced around the original position. However, the amplitude of displacement and the range of movement between each time interval of 20 min for normal HepG2 cells were evidently higher than those for HepG2 cells incubated with EGCG. Moreover, the EGCG-treated cells were more likely to vibrate continuously rather than displace for a significant distance. With the help of cell migration tracking assay, the stack of phase-contrast images against the time of cell culture was further analyzed.

Figure 4.7 The overlay of phase-contrast images (at time 0 and 4 hours) of the migration tracks (the redline) of normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm-grafted surface during 16 hours of incubation at 37 °C and 5% CO_2 .

The scale-bar represents 100 μm .

4.6 Persistence random walk analysis of cell migration under EGCG

treatment

The persistent random walk model we applied in this study is known as a best model to quantify cell migration on biomaterial surface [146]. Fig. 4.8 A showed the root mean-square displacement $\langle d(t)^2 \rangle$ against time for at least 50 normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm surface during 16 h of culture at 37 °C and 5% CO_2 . $\langle d(t)^2 \rangle$ of HepG2 cells treated with EGCG was significantly lower than that of normal cells throughout the whole time course. After 16 hours, $\langle d(t)^2 \rangle$ of HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG reached 159.09 μm^2 and 70.57 μm^2 respectively, which were 63.3% and 83.72% lower than those of normal cells (433.40 μm^2). The averaged instantaneous speed for each type of cells was calculated from the slope and intercept of the trendline (the dot line) of $\langle d(t)^2 \rangle$ vs. t curve.

(A)

(B)

Figure 4.8 (A) the root mean-square displacement $\langle d(t)^2 \rangle$ against time t of at least 50 different types of HepG2 cells on laminin-coated PIPAAm surface. The dot lines were the trendlines (the dot line) of $\langle d(t)^2 \rangle$ vs. t curves. (B) The persistence time (P) vs. root mean-square speed (S) of at least 50 different types of HepG2 cells on laminin-coated PIPAAm surface.

The instantaneous speed of each cell is calculated from the slope and intercept of the trendline of $\langle d(t)^2 \rangle$ vs. t curve.

Fig. 4.8 B showed the persistence time (P) and root mean-square speed (S) of at least 50 normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm surface during 16 h of culture at 37 °C and 5% CO₂. The persistence time of HepG2 cells incubated with 5 $\mu\text{g/ml}$ (258.16 min) and 7.5 $\mu\text{g/ml}$ of EGCG (452.22 min) were conspicuously higher than that of normal cells (200.66 min). The result indicated that EGCG enhanced frequency in the change of migration directions of HepG2 cells. In addition, the root mean-square speed of normal HepG2 cells, HepG2 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG were 0.0383, 0.0208 and 0.0124 $\mu\text{m/min}$, respectively. The difference as mentioned above between normal cells and cells treated with EGCG was verified by paired Student's t -test ($p < 0.05$). Hence, synchronous alteration in both cell persistence time and motility was elicited by the interaction with EGCG. It was feasible to claim that EGCG inhibited the normal cell movement in a dose-dependent fashion.

Cell migration tracking assay was also carried out together with the use of Meta Morph 7.0 software to track the movement of the different types of HepG2 cells treated with different concentration of EGCG over the duration of 16 hours. From the images taken, it is evident that HepG2 cells not treated with any EGCG showed higher degree of migration in the 16 hours as compared to cells treated with EGCG. Furthermore, from the various graphs plotted regarding the RMS migration speed of the cells, an obvious trend was observed. The rate of migration of the HepG2 cells decreases with increasing concentration of the EGCG which once again agrees with the assumption that EGCG inhibits cell migration and affects the spreading kinetics of the cells [160].

4.7 Discussion & Conclusion

This project provides an extensive study on the effect of EGCG on the contact mechanics, receptor-mediated adhesion/deadhesion dynamics and spreading kinetics of a type of hepatoblastoma-derived cell line, HepG2 cells. A variety of microscopic techniques and software were utilized to carry out the various experiments. Furthermore, a type of TRP, PIPAAm was also used to facilitate cell adhesion and cell deadhesion at different temperature. This enables the monitoring of the evolution of the contact mechanism of the HepG2 cells across a temperature change.

To date, the antiproliferative effects of EGCG on cancer prevention and inhibition has been widely proven in previous studies. Nevertheless, the consequences of EGCG treatment on cell migration-related activities on extracellular matrix or biomaterial remained to be vague. Generally, tumor invasion and metastasis could be induced by the deregulation or moderation of cell migration [151]. In this study, it was generally shown that there is a tight coupling between cell adhesion/deadhesion kinetics, migration parameters and EGCG treatment on ECM-coated TRP surface. In order for a cancerous cell to trigger movement from the original site, it is necessary to down-regulate adhesion and up-regulate deadhesion. To migrate, a cell first extends protrusions such as lamellipodia and filopodia, through deadhesion and adhesion of the front end, and finally retracts its tail through a synergistic cascade of adhesion and deadhesion. Hence the correlation between adhesion/deadhesion kinetics and cell migration parameters of HepG2 cells under EGCG treatment as reported herein supported the possible role of EGCG in interfering and preventing the metastasis of liver cancer.

Taken together, by the combination of adhesion/deadhesion assay, molecular biology technique, and real-time cell migration tracking assay, we investigated the effect of EGCG on the adhesion/deadhesion kinetics, assembly/disassembly of actin filaments and cell migration of HepG2 cells on model ECM-coated TRP. The acquired results indicated that EGCG tuned all the

biophysical features of adhesion/deadhesion in a dose-dependent manner. It was also proven that EGCG played a crucial role in the dynamic cell migration flow. Cell migration was shown to be inhibited or decelerated by EGCG treatment. Our biophysical approaches integrated with the thermo-responsive PIPAAM system was substantiated to be a highly quantitative assessment system for investigating intricate cellular responses to external stimuli including nominees of therapy.

Chapter 5 The restoration of the abnormal cell migration of HepG2.2.15 under EGCG treatment

In previous chapters, adhesion contact dynamics, deadhesion kinetics and real-time migration of HepG2 cells under HBV replication and EGCG treatment were thoroughly investigated. Interestingly, the effect of HBV replication on the dynamic cell adhesion/deadhesion and migration was opposite to that of EGCG treatment. Nevertheless, the direct impact of EGCG on cancer cells affected by HBV replication remains unknown

5.1 The validation of HBV replication in HepG2.2.15 cells

The results we have acquired so far were based on the transfection of HBV containing plasmids into HepG2 cells. The transfection was mainly a transient over-expression of HBV genes. The transient transfection was an effective and widely used method to detect the impact of the protein expressions. However, HepG2 cells transfected with HBV genome were unstable. The expression level was significantly lower than that caused by HBV infection. It was difficult to steadily passage the transfected cells due to the exocytosis of plasmids after several generations. To fully simulate the actual HBV infection to the greatest extent, the stable cell line would be needed. Hence, HepG2.2.15, a stable cell line derived

from HepG2, containing twice head-to-tail of HBV genome [148, 165, 166], was introduced in this part of my study in order to mimic the abundant HBV replication found in HBV infected cells.

IMX offers a qualitative and semi-quantitative measurement of the HBV replication activity. IMX has been widely used in clinical diagnosis for the identification of chronic HBV carriers. In this system, Microparticle Enzyme Immunoassay (MEIA) technology was used for the detection of Hepatitis B surface antigen (HBsAg) in order to verify sufficient HBV replication in HepG2.2.15 cells. As shown in Figure 5.1, HepG2.2.15 significantly releases HBsAg, whereas HepG2 alone showed insignificant amount of HBsAg. On the other hand, HepG2 cells transfected with HBV genome can also release HBsAg, albeit the S/CO ratio was only around one third of that of HepG2.2.15. Hence, HepG2.2.15 was proven to be an extreme and stable system for HBV replication compared to HBV transfected HepG2 cells.

Figure 5.1 Secreted HBsAg detection by IMX. Data were presented as the S/CO ratio (Y-axis), referring to the Sample /Cut-Off Rate, which was autogenerated and provided by the system. S/CO ratio >1 was taken to be reactive and S/CO ratio <1 was taken to be non-reactive. The positive and negative controls were reagents originally provided together with IMX kit.

5.2 Cell migration profile of HepG2.2.15 under EGCG treatment

By employing our real-time cell migration assay as described previously, the migration parameters of HepG2.2.15 cells under the concomitant HBV replication and EGCG treatment was probed from a series of phase contrast images. Figure 5.2 showed the typical migration tracks (the redline) on the overlay of a pair of phase contrast images (at time 0 and 16 hours) of normal HepG2.2.15 cells, HepG2.2.15 cells incubated with 5 μ g/ml of EGCG and HepG2.2.15 cells

incubated with 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm-grafted surface during 16 hours of incubation at 37 °C under 5% CO_2 atmosphere. It was observed that all three types of HepG2.2.15 cells moved away from the original positions. However, the extent of displacement during each time interval of 20 min for HepG2.2.15 cells incubated with the two different concentrations of EGCG were apparently lower than that for normal HepG2.2.15 cells. Furthermore, the EGCG-treated cells were more likely to vibrate continuously rather than displace in a certain direction and for a significant distance. The acquired stack of phase-contrast images against the time of cell culture was further analyzed with the utilization of cell migration tracking assay.

Figure 5.2 The migration tracks (the redline) on the overlay of two phase-contrast images (at time 0 and 16 hours) for normal HepG2.2.15 cells, HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ and HepG2.2.15 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm-grafted surface during 16 hours of incubation at 37 °C and 5% CO_2 .

5.3 Persistence random walk analysis of HepG2.2.15 cell migration under EGCG treatment

The quantitative analysis of the migration of HepG2.2.15 cell was performed with persistent random walk model. Figure 5.3 A showed the root mean-square displacement $\langle d(t)^2 \rangle$ against time for at least fifty normal HepG2.2.15 cells, HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ of EGCG and HepG2.2.15 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm surface during 16 h of culture at 37 °C and 5% CO₂. $\langle d(t)^2 \rangle$ of HepG2.2.15 cells treated with EGCG was remarkably lower than that of normal HepG2.2.15 cells throughout the whole period. After 16 hours, $\langle d(t)^2 \rangle$ of HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ and 7.5 $\mu\text{g/ml}$ of EGCG reached 2658.20 μm^2 and 1827.41 μm^2 respectively, which was 57.80% and 70.99% lower than that of normal HepG2.2.15 cells (6298.77 μm^2). The averaged instantaneous speed for each type of cells was calculated from the slope and intercept of the trendline (the dot line) of $\langle d(t)^2 \rangle$ vs. t curve. Figure 5.3 B showed the persistence time (P) and root-mean-square speed (S) of at least fifty normal HepG2.2.15 cells, HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ and HepG2.2.15 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG on laminin-coated PIPAAm surface during 16 h of culture at 37 °C and 5% CO₂. The persistence time of HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ (107.31 min) or 7.5 $\mu\text{g/ml}$ of EGCG (107.82 min) was significantly higher than that of normal Hep2.2.15 cells (98.28 min). The result indicated that EGCG reduced the frequency in the change of migration directions of HepG2.2.15 cells. In addition, the root-mean-square speed

of normal HepG2.2.15 cells, HepG2.2.15 cells incubated with 5 $\mu\text{g/ml}$ and HepG2.2.15 cells incubated with 7.5 $\mu\text{g/ml}$ of EGCG was 0.2609, 0.1606 and 0.1410 $\mu\text{m/min}$, respectively. The statistical significance on the difference of P and S between normal cells and cells treated with EGCG as mentioned above was further verified by paired Student's t -test ($p < 0.05$). Therefore EGCG was effective in the shift of both cell persistence time and speed. It was logical to assert that EGCG suppressed the otherwise abnormally high motility of HepG2.1.15 cells in a dose-dependent manner.

(A)

(B)

Figure 5.3 (A) Root mean-square displacement $\langle d(t)^2 \rangle$ against time t of at least fifty different types of HepG2.2.15 cells on laminin-coated PIPAAm surface. The dot lines were the trendlines (the dot line) of $\langle d(t)^2 \rangle$ vs. t curves. (B) The persistence time (P) vs. root mean-square speed (S) of at least fifty HepG2.2.15 cells with different treatments on laminin-coated PIPAAm surface. The instantaneous speed of each cell was calculated from the slope and intercept of the trendline of $\langle d(t)^2 \rangle$ vs. t curve.

5.4 Discussion & Conclusion

After exploring the relation between adhesion/deadhesion kinetics and cell migration parameters of HepG2 cells under EGCG treatment, we demonstrated the

potential role of EGCG in modulating the metastasis of liver cancer. Previously, we also accomplished the identical work on HBV genome transfected HepG2 cells. Nevertheless, the direct impact of EGCG treatment for HBV infection remains obscure. Thus we introduced HepG2.2.15 cell line in order to provide a tailored cell culture system with stable and sufficient HBV replication. With the utilization of real-time cell migration tracking assay, we investigated the effect of EGCG on the abnormally elevated motility of HepG2.2.15 cells on model ECM-coated TRP. The results suggested that EGCG restored the high cell motility of HepG2.2.15 towards the original level of normal HepG2 cells in a dose-dependent fashion. It was again testified that EGCG played a decisive role in the inhibition of cell migration. Through probing intricate cellular responses to external stimuli, the quantitative function of our biophysical approaches applied herein was proven to be good at evaluating new and potential cancer therapeutics.

Chapter 6 Protein profile in HepG2 cells tranfected with HBV genome using iTRAQ coupled LC-MS/MS

Wild type and mutant HBV genome were cloned into mammalian expression vector pcDNA3.1 and verified by DNA sequencing. The mutation introduced in proline-rich region in SH3 binding domain of HBx may elicit a structural and in turn functional alteration as most protein-protein interactions are based on the matching of conformation. The proline-rich domain interacts with SH3 domain and participates in various cellular events. In previous studies, our group has demonstrated that this region can indeed interact with SH3 domain [63].

To simulate the early HBV infection and characterize early cellular responses to HBV infection specifically associated with the proline-rich region in SH3 binding domain, HepG2 cells were transfected with wild type and mutant HBV genome cloned in pcDNA3.1 vector. The comprehensive protein profile indicating global cellular events in response to transfection with HBV genome was in turn analyzed by the high throughput, broad-spectrum and sensitive iTRAQ-coupled LC-MS/MS analysis.

The expression of HBV genome was previously validated by reverse transcript-PCR (Fig. 3.2), The result indicated that the wild type or mutant pcDNA3.1-HBV was successfully expressed in HepG2 cells.

6.1 iTRAQ analysis and differentially expressed proteins

As described in 2.13.2, BSA was used as a standard exhibiting the representative spectrum to justify the feasibility of the iTRAQ approach [137]. To investigate the protein profile in the HepG2 cells transfected with wild type and mutant HBV genome compared to those transfected with empty pcDNA3.1 vector, the iTRAQ was used to label as following: HepG2 cells transfected with empty vector pcDNA3.1 = iTRAQ 114; HepG2 cells transfected with wild type HBV genome = iTRAQ 116; HepG2 cells transfected with mutant HBV genome = iTRAQ 117. In each of the three independent experiments, the total proteins were extracted from the transfected cells and labeled with iTRAQ reagents on the same assignments of the isobaric ions. Spectrum Mill was used to identify and quantify the different protein expression levels among different samples. Figure 6.1 showed a representative MS/MS spectrum of the peptide HVGMAVAGLLADAR from the protein of PSMA3. It showed that the same peptide was up-regulated in the samples labeled by iTRAQ 116 and 117 which was consistent with the change of protein expression level.

A total of 169 unique proteins were identified in three independent experiments as mentioned above and standard errors as shown in table 6.1 were calculated from the three independent experiments. Identification and quantification of proteins with significant expression level were selected by

Spectra Mill software with the Distinct Summed MS/MS Search Score of more than 7.0, which gave the confidence value of 99% and one or more peptide hits were found per protein at > 95% confidence per peptide. Other identified proteins with Distinct Summed MS/MS Search Score less than 7.0 were filtered for further analysis. Among the 169 identified proteins, 24 differentially expressed proteins were selected herein and sorted into four major groups according to their cellular functions. In general, 5 metabolic proteins, 11 signaling proteins, 6 cytoskeletal proteins and 2 cell growth related proteins were shown in Table 6.1.

Table 6.1 List of differentially expressed cellular proteins

DSMS ^{a)} score	Accession no. and protein name and	avg. wt:pc ^{b)}	avg. m:pc ^{c)}	Function ([167] and references therein)
metabolic enzymes				
148.60	(P00558) Phosphoglycerate kinase 1	1.43±0.13	1.23±0.02	A polymerase alpha cofactor protein (primer recognition protein)
60.38	(P31327) Carbamoyl-phosphate synthetase I	1.40±0.06	1.13±0.06	Involved in the urea cycle of ureotelic animals where the enzyme plays an important role in removing excess ammonia from the cell.
79.02	(P62937)Peptidyl-prolyl cis-trans isomerase A	1.20±0.05	1.10±0.03	PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.
241.36	(P06733) Alpha-enolase	1.29±0.07	1.15±0.04	Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses.
Signaling proteins				
105.39	(P07355) Annexin A2	1.26±0.12	1.15±0.17	Calcium-binding protein
	(P09651) hnRNP A1	1.16±0.06	1.17±0.10	Involved in the packaging of pre-mRNA into hnRNP particles, transport of poly(A) mRNA from the nucleus to the cytoplasm and may modulate splice site selection
40.80	(P22626) hnRNP A2/B1	1.09±0.05	1.09±0.00	Involved with pre-mRNA processing
41.82	(P61978) hnRNP K	1.28±0.09	0.95±0.06	Binds to pre-mRNA. Binds tenaciously to

				poly(C) sequences. Likely to play a role in the nuclear metabolism of hnRNAs
32.59	(Q00839) hnRNP U	1.11±0.13	0.97±0.06	Binds to pre-mRNA. Has high affinity for scaffold-attached region (SAR) DNA. Bind to double- and single-stranded DNA and RNA
67.17	(P27797) Calreticulin	1.22±0.03	0.97±0.04	Molecular calcium binding chaperone promoting folding, oligomeric assembly and quality control in the ER via the calreticulin/calnexin cycle.
71.07	(P06703) Protein S100-A6	0.93±0.05	0.96±0.01	Calcium-binding protein
17.99	(P62826) GTP-binding nuclear protein Ran	1.47±0.24	1.04±0.19	GTP-binding protein involved in nucleocytoplasmic transport.
49.37	(P05161) Interferon-induced 17 kDa protein	1.37±0.13	1.24±0.14	Ubiquitin-like protein that is conjugated to intracellular target proteins after IFN-alpha or IFN-beta stimulation.
56.12	(Q06830) Peroxiredoxin-1	0.93±0.04	0.90±0.10	Involved in redox regulation of the cell
167.23	(P11142) Heat shock cognate 71 kDa protein	1.32±0.06	1.20±0.02	Endogenous inhibitory regulator of HSC70 by competing the co-chaperones
Cytoskeleton proteins				
160.50	(P08670) Vimentin	1.27±0.01	1.16±0.04	Class-III intermediate filaments found in various non-epithelial cells
211.12	(P07437) Tubulin beta chain	1.47±0.09	1.37±0.06	The major constituent of microtubules.
59.64	(P02545) Lamin-A/C	1.21±0.03	1.19±0.14	Components of the nuclear lamina
13.55	(O43707) α -actinin-4	2.20±0.72	1.39±0.18	Anchor actin to a variety of intracellular structures
212.77	(P05787) Keratin-8	1.23±0.02	1.18±0.01	Link the contractile apparatus to dystrophin at the costameres of striated muscle.
100.16	(P06748) Nucleophosmin	1.30±0.15	1.30±0.15	Involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors TP53/p53 and ARF.
Cell growth-related proteins				
54.13	(Q5VTE0) Putative elongation factor 1-alpha-like 3	1.36±0.06	1.21±0.04	Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.
18.52	(P26641) Elongation factor 1-gamma	0.84±0.08	0.92±0.06	Probably plays a role in anchoring the complex to other cellular components.

- Distinct Summed MS/MS Search Score
- the ratio of differential protein expression level in the protein extracts from the cells transfected with wild type HBV genome to the protein extracts from the

- cells transfected with empty pcDNA3.1
- c) the ratio of differential protein expression level in the protein extracts from the cells transfected with mutant HBV genome to the protein extracts from the cells transfected with empty pcDNA3.1

The standard errors were generated from three independent experiments, and in each experiment the protein extractions were prepared and labeled with iTRAQ reagents with the same arrangement of the isobaric ions.

6.2 Differentially expressed proteins involved in cell migration

Most cytoskeleton proteins as shown in Table 6.1 were found to be up-regulated by the HBV genome expression. Vimentin, a major component of intermediate filament, has been reported to be over-expressed in various cancer cells [168], whereas tubulin beta chain and lamin-A/C are respectively major constituents of microtubules [169] and nuclear lamina [170]. α -actinin-4 anchors actin to a variety of intracellular structures [171] while keratin-8 links the contractile apparatus to dystrophin at the costameres of striated muscle [172]. Nucleophosmin is involved in diverse cellular processes such as ribosome biogenesis, centrosome duplication, protein chaperoning, histone assembly, cell proliferation, and regulation of tumor suppressors TP53/p53 and ARF [173, 174].

Among all the listed up-regulated proteins in the group of cytoskeleton proteins, vimentin is the most remarkable one. Vimentin is a ubiquitous

mesenchymal intermediate filament serving as scaffold and anchor of the cytosolic organelles as well as participating in cell adhesion, migration, survival, and signaling pathways through dynamic assembly/disassembly in activated cells. It is laterally or terminally attached to the nucleus, mitochondria and endoplasmic reticulum [175]. Moreover, the impact of vimentin on tumor growth *in vitro* and *in vivo* and the role of vimentin in drug-induced responses have been being widely evaluated and explored.

The flexibility of cell is greatly attributed to the dynamic nature of vimentin. It has been reported that vimentin provided cells with a resilience in the absence of the actin filament networks or microtubule under *in vivo* mechanical stress. Therefore, it is generally acknowledged that being a component of cytoskeleton, vimentin is responsible for integrating cytoplasm, maintaining cell shape, and stabilizing cytoskeletal interactions. Cells without vimentin were found to be extremely fragile under the disturbance with a micropuncture [176].

Interestingly, it has also been revealed that vimentin participate in the regulation of cell migration and in turn tumor invasion and metastasis [177]. The formation of metastasis in distant organs is a critical step in cancer development and a major cause of mortality. To escape from the original tumor site and invade

into adjacent tissues, cancer cells must first degrade the basement membrane that separates the epithelial and stromal compartments [178]. The degradation of the basement membrane is mediated by matrix metalloproteinases (MMPs). In cell culture assays, MMPs accumulate in invadopodia, finger-like membrane protrusions which develop on the ventral surface of cancer cells [179-181]. The actin polymerization machinery is vital for the development and function of the actin-rich invadopodia.

Vimentin filaments have been shown to be present in podosomes of macrophages [182]. Nevertheless, no intermediate filaments have been detected in premature invadopodia, suggesting the structural distinctness between these two organelles. The presence of keratin and vimentin in mature invadopodia and the necessity of an intact vimentin network for invadopodia elongation have been validated. Hence it is well corroborated that intermediate filaments played an essential role in regulating cell shape and tissue homeostasis. Vimentin expression and a switch in keratin expression has been associated with the epithelial-mesenchymal transition and cell-cell adhesions during cancer invasion [183, 184]. Furthermore, it was reported that vimentin is required for the motility and invasion of prostate cancer cells [185]. Therefore, it is justified to claim that vimentin participate in regulating cell invasion via mechanically stabilizing mature invadopodia.

In our comparative proteomics analysis, vimentin was found to be up-regulated by 27% in HepG2 cells transfected with wild-type HBV genome, whereas the up-regulation was recovered to 16% in HepG2 cells transfected with mutant HBV genome. Since vimentin is highly associated with the formation of invadopodia and thus enhances the tendency to migrate from the original site, the up-regulation of vimentin by wild-type HBV genome replication was consistent with the enhanced cell adhesion and migration that we acquired by contact kinetics analysis and cell migration assay, while the recovery of up-regulation by mutation in proline-rich region agreed well with the restoration to normal cell adhesion and motility profile.

In the group of cytoskeletal proteins, keratin-8 was also found to be up-regulated in HepG2 cells transfected with wild type HBV genome but slightly restored in HepG2 cells transfected with mutant HBV genome. It has been reported that the up-regulation of keratin-8 was only detected in p21-HBx but not p21-HBsAg liver tissues from transgenic mice, suggesting that the global change in the expression level of cellular cytoskeletal proteins was associated with the expression of HBx transgene [186].

Keratin-8 and keratin-18 are the two major cytoplasmic intermediate

filaments (IFs) distributing in adult hepatocytes. Keratin-8 and keratin-18 extend from the nuclear surface to the cell periphery in hepatocytes [187] and the C-terminal end of keratin-8 penetrates the cell membrane in hepatocellular and breast carcinoma cells [188, 189]. Although IFs has been known for its strong mechanical strength, it has been shown by the assessment of their assembly and dynamics in live cells that they alone cannot form a static cytoskeletal network [190, 191]. Keratin-8/keratin-18 provide resistance not only to various forms of stress applied to the cell surface but also to apoptosis in simple epithelial cells, like hepatocytes. Mechanical stress appears to overwhelm mechanoreceptors anchored in cell membrane, such as integrins and cadherins, which are mechanically coupled with cytoskeletal networks, particularly keratin IFs, and even nuclear scaffolds. The absence of keratin-8 and keratin-18 in hepatocytes resulted in an accelerated cell adhesion to fibronectin compared with wild-type hepatocytes [192]. Keratin IFs constitute a highly dynamic cytoskeletal network, and their functions are correlated with proper movement and dispatching at corresponding targeted cell membrane sites.

The versatile roles of keratin-8 appear to be mainly contributed by the head and tail domains. It has recently been identified that the monoclonal antibody that recognizes an epitope in ectoplasmic domain of keratin-8 inhibits plasminogen activation mediated by urokinase-type plasminogen activator (uPA) in breast cancer cells [193]. The generation of plasmin is a hallmark of tumor cells,

promoting the degradation of extracellular matrix tumor development and metastasis. The process is accelerated by the binding of plasminogen, plasminogen activator and their cell membrane receptors [194].

These results implied a system in which keratin-8, together with uPA, plasminogen and ECM protein, constitutes a signaling network for modulating cell adhesion/migration/growth-dependent signal transduction. Anti-keratin monoclonal antibody, which competes with uPA for the binding site, has the potential to be used as an effective agent to reduce the metastatic and invasive potential of cancer cells [193]. Our finding on the up-regulated keratin-8 in HepG2 cells transfected with wild type HBV leading possibly to enhanced cell migration was consistent with the reported association between HBV and HCC. Furthermore, the slight restoration of HepG2 cells transfected with mutant HBV attributes to the proline-rich region in SH3-binding domain of HBx into the modulatory network.

In the group of signaling proteins, several proteins including calreticulin and annexin A2 were up-regulated. Calreticulin is an ample and versatile calcium-binding protein located in the endoplasmic reticulum lumen of most human cells. Through binding to misfolded proteins, calreticulin plays as an vital quality control role in the synthesis and folding processes of protein [195]. Calreticulin has also been involved in various cellular behavior outside the endoplasmic reticulum, including integrin-mediated calcium signaling, cell

adhesion and expression of steroid-sensitive genes [196]. It has been widely reported that calreticulin enhances wound healing and cell migration [197], and regulates apoptosis via the modulation of intracellular calcium stores [198-200]. Akt activation has also been shown to play an essential role in calreticulin-correlated apoptosis [201-203]. Recent work has proven that calreticulin protects fibroblasts from apoptotic cell death induced by cell detachment via binding to TSP-1 and LRP1 [204]. Embryonic lethal characteristics were induced in calreticulin-deficient mice due to the alteration of cell adhesiveness and the impairment of cardiac function and development [198, 205, 206].

Calreticulin may be implicated in the progression and invasion of a variety of human cancers: up-regulation of calreticulin has been found in hepatocellular carcinoma [207], breast carcinoma [208, 209], prostate adenocarcinoma [210] and colon cancer [211]. It has been revealed that the overexpression of calreticulin is a prevalent event in malignant transformation of esophageal epithelial cells [212, 213]. Anoikis, the apoptotic cell death due to loss of cell adhesion, is known to be pivotal in the regulation of tissue homeostasis in tumor progression, invasion, metastasis, fibrosis and tissue remodeling [214]. Recently, it has been validated that calreticulin promotes cell motility and thus enhances resistance to anoikis in esophageal squamous cell carcinoma through a cortactin-associated pathway [215]. All results as mentioned above are in line with the possible role of proline-rich

region of HBx in the up-regulation of calreticulin by 22% in HepG2 cells transfected with wild type HBV genome and the complete restoration in HepG2 cells transfected with mutant HBV genome.

The up-regulated annexin A2 in our study, a protein belonging to a widely distributed annexin family, is closely related to calcium and membrane binding. The up-regulation of annexin A2 has been found in several types of tumor, gastric adenocarcinoma [216], colorectal adenocarcinoma [217, 218], kidney cancer [219], pancreatic adenocarcinoma [220], and HCC [221]. Interestingly, the up-regulation of annexin A2 has been reported in the HBV replicative cell line HepRG recently [222]. This is consistent with our results on the up-regulation of annexin A2, which also regulates plasmin generation [223-228], and has been implicated in many biological processes involving cell migration, such as tissue remodeling, angiogenesis, tumor development and invasion [229, 230]. Taken together, the up-regulation of annexin A2 by HBV genome replication and HBx expression may be involved in the development of HCC as well.

Cell migration is a dynamic and highly organized behavior involving not only actin but also a large group of actin-binding proteins. The proteins identified in our study were mostly up-regulated in HepG2 cells transfected with wild type HBV genome and restored more or less by the mutation introduced in proline-rich region in SH3 binding domain of HBx. Their up-regulations may impact on the

cytoskeleton organization and cell migration, either directly or indirectly.

The S100 proteins belong to the calcium-binding EF-hand motif superfamily comprising 20 known human members. S100 proteins possess a broad range of intracellular and extracellular functions. Increased expression of S100A6 in human osteosarcoma cells were found to be associated with decreased metastasis and inhibition of cell migration and anchorage independent growth on collagen gels [231]. The down-regulation of S100A6 found in our study (Table 6.1) was consistent with our findings based on adhesion contact kinetics and cell migration assay (Chapter 3), as well as the recently published results through the analysis of the HBV replicative cell line HepG2.2.15 [232].

6.3 Conclusion

To sum up, our results suggested that the iTRAQ LC-MS/MS based quantitative proteomics analysis is an effective approach to investigate the comparative protein profile in HepG2 cells under HBV genome expression. A series of differentially expressed proteins were identified, some of which were highly relevant to cell migration. The integrated effects of these differentially expression proteins may provide valuable information on the underlying mechanism of the function of HBx in HCC, as well as HBV-associated HCC and the specific interaction between HBx and vinexin β [147]. The selected proteins

like vimentin, keratin-8, calreticulin, annexin A2 and S100A6 may also serve as useful biomarkers indicating early stage of cancer development.

Chapter 7 Conclusion

It has been demonstrated that the temperature transition across the LCST triggered the deadhesion of the various types of HBV genome transfected HepG2 cells from the TRP PIPAAm due to the change of the TRP surface from hydrophobic to hydrophilic. In addition, it was shown that the impact of SH3 binding domain in HBX gene plays an essential role on the deadhesion and migration process. The intact SH3 binding domain presents in HepG2 cell transfected with wild type HBV genome blocked the focal adhesion formation pathway which was occurring at the same time as the deadhesion and migration process. This further increased the rate of deadhesion and migration speed from TRP below LCST. Among the three types of HepG2 cells, both the mutant HBV and empty vector transfected HepG2 cells do not contain any functional SH3 binding domain, thus their rates of deadhesion and migration speed were lower as compared to HepG2 cells transfected with wild type HBV genome and comparable with each other. The hypothesis was supported by the results of key biophysical parameters including average normalized contact areas, degree of deformation, average adhesion energy against time and RMS migration speed. All the parameters of the HepG2 cells transfected with wild type HBV decreased the fastest with time, whereas the parameters of the other two types of HepG2 cells decreased at a lower rate. It was the presence of intact SH3 binding domain of HBX gene in HBV genome that led to the faster rate of deadhesion and highest cell migration speed as it was able to block the focal adhesion formation pathway.

Confocal and phase contrast microscopy techniques had also been successfully applied in determining the effect of EGCG on adhesion/deadhesion kinetics of HepG2 cells on a laminin coated substrate. The results showed that increasing concentration of EGCG accelerated/decelerated the rate of adhesion/deadhesion. The cell migration speed was also affected due to the inhibitory effect of EGCG on the cell motility of HepG2 cells. The increase in EGCG concentration also accelerated the increase in degree of cell deformation, which reflected the change in rate of cell deformation. Rate of average adhesion energy was accelerated with the increase in concentration of EGCG and was consistent with the findings from the degree of cell deformation. Overall, EGCG affected the receptor-mediated adhesion between HepG2 cells and the laminin-coated TRP surface, which has been shown to consequently affect the cell migratory behaviour of cells. Furthermore, a critical cell culture with high HBV replication, HepG2.2.15, was introduced to investigate the direct interaction of EGCG and HBV replication regarding cell migration behavior. The results demonstrated that EGCG may possibly be a promising candidate to treat cancer invasion. Studies on the effect of EGCG on HBV replication-induced liver cancer development can be performed in the future to further support the results of this study. Taken together, the results obtained from this project were all in agreement with the other studies conducted and further proved EGCG's modulatory effect on cell adhesion/deadhesion and migration [160]. Moreover, it provided an insight on the therapeutic effect EGCG may potentially possess against cancer cells invasion

and metastasis [233].

After exploring the relation between adhesion/deadhesion kinetics and cell migration parameters of HepG2 cells under EGCG treatment, we demonstrated the potential role of EGCG in modulating the metastasis of liver cancer. Previously, we also accomplished the identical work on HBV genome transfected HepG2 cells. Nevertheless, the direct impact of EGCG treatment for HBV infection remains obscure. Thus we introduced HepG2.2.15 cell line in order to provide a tailored cell culture system with stable and sufficient HBV replication. With the utilization of real-time cell migration tracking assay, we investigated the effect of EGCG on the abnormally elevated motility of HepG2.2.15 cells on model ECM-coated TRP. The results suggested that EGCG restored the high cell motility of HepG2.2.15 towards the original level of normal HepG2 cells in a dose-dependent fashion. It was again testified that EGCG played a decisive role in the inhibition of cell migration. Through probing intricate cellular responses to external stimuli, the quantitative function of our biophysical approaches applied herein was proven to be good at evaluating new and potential cancer therapeutics.

Our comparative proteomics results suggested that the iTRAQ LC-MS/MS based quantitative proteomics analysis is an effective approach to investigate the comparative protein profile in HepG2 cells under HBV genome expression. A series of differentially expressed proteins were identified, some of which were

highly related with cell migration. The integrated effects of these differentially expression proteins may provide valuable information on the underlying mechanism of the function of HBx in modulation of cellular behavior. The selected proteins like vimentin, keratin-8, calreticulin, annexin A2 and S100A6 may also serve as nominees of useful biomarkers.

Chapter 8 Future Work

8.1 Intracellular signal pathway in HBV infected liver cancer cells and the effects on ECM characteristics

During cell migration and tumor metastasis, the ECM proteins play important role in carcinoma development [234]. Thus cellular signaling cascades mediated by ECM protein could represent potential targets for the development of new anticancer drugs.

In my thesis, I have elucidated the biophysical basis of cellular responses caused by the HBV protein expression. By comparing the wide type HBV and mutant HBV, it offered us a clue on how the transfection of HBV genome can moderate cell adhesion at the molecular level. We have elucidated the effect of HBV infection on the cellular responses but the underlying signal pathway still remains unknown. The next step is to find out the interaction between the intracellular proteins and HBx via involving the proline-rich domains. The interactions between HBx and some intracellular proteins have been shown by commercial SH3 binding array in some previous work [235]. Nevertheless, the exact proteins involved in the cancer cell motility and tumor metastasis remain to be determined. Based on the work from the literature, a series of signal

transduction pathways related to cell proliferation, survival and migration has been largely established. Combining the HBx protein characterizations and existent pathways, we will aim to find out the pathway involving HBV. The proline-rich domain (SH3 binding domain) may play a key role in the signaling pathways induced by HBV infection. Also, the different ECM proteins will be focused on for their specificity of receptor binding.

8.2 Further comparative iTRAQ-coupled LC-MS/MS proteomics analysis of liver cancer cells under HBV replication, EGCG treatment or other external stimuli

Cytoskeleton remodeling is commonly associated with HBV-induced cancer development and tumor metastasis, which is a multi-step process [73]. To date, many proteins involved in cell migration and metastasis have been identified in various liver cancer cell lines or clinical samples. Moreover, many proteins involved in cell growth, metabolic enzymes and signaling pathway, etc. were distinguished from the proteome to be regulated and modulated by HBV replication and EGCG treatment.

It is generally known that the protein expression of a cell is a highly dynamic parameter which is altered by internal, external stimuli and physiological condition. Previous proteomics analysis has been applied to elucidate the

molecular basis of several liver diseases such as cirrhosis and HCC [137, 236, 237]. Nowadays the iTRAQ-coupled LC-MS/MS analysis has been applied to demonstrate and quantify the differences in protein expression levels of normal cells and drug-treated cells. It is more effective and sensitive than the traditional 2D-gel electrophoresis coupled with MS identification [137, 236, 237].

The results we acquired before with the comparative proteomics approach above has demonstrated that HBx modulated the expression levels of proteins involved in metabolic enzymes, signaling pathway and cytoskeleton regulation [235], also identified several proteins regulating cell migration. Thus the cell culture based assay was ready to probe the early cellular response to HBx expression during HBV replication and EGCG treatment.

Similarly to other analysis on intracellular profile in HBV replicating cells, RT-PCR and Western blot analysis could be applied to validate the differential expression profile identified in LC-MS/MS.

To sum up, the application of iTRAQ-coupled 2D LC-MS/MS based quantitative proteomics analysis would be an operative approach to investigate the protein profile of HepG2 cells (or HepG2.2.15 for critical HBV replication) under HBV replication or EGCG treatment. Some of the identified differentially expressed proteins may be highly associated with cell adhesion/deadhesion,

migration and in turn cancer invasion and metastasis. In addition, the changes in these proteins involved in cell migration could be verified by the *in vitro* cell adhesion/deadhesion kinetics and migration assay. The comprehensive interplay of these differentially expressed proteins may illustrate the underlying mechanism regarding SH3 binding domain in HBx, HBV replication, HBx-protein and EGCG-protein interaction.

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