INVESTIGATIONS OF THE NEW CELL ADHESION MOLECULE HEPACAM: ITS CONNECTION WITH INTEGRIN-DEPENDENT SIGNALING AND ITS PROTEOLYTIC CLEAVAGE

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SUMMARY

Cell adhesion is a fundamental feature of multi-cellular organisms. It is crucial not only for the formation and maintenance of cellular architecture but also for the normal biological processes. Cell-cell and cell-extracellular matrix (ECM) adhesions are mediated by a large number of cell adhesion molecules (CAMs) which are classified into five major families: immunoglobulin (Ig)-like CAMs, integrins, selectins, cadherins and heparin sulfate proteoglycans (HSPG).

Previously, a novel gene *hepaCAM* in liver was identified by Dr. Shen Shali’s group. Mapped to human chromosome 11q24, *hepaCAM* encodes a CAM of the Ig-like superfamily. The gene is widely expressed in normal human tissues and is frequently silenced in a variety of tumors. Re-expression of hepaCAM in cancer cell lines significantly inhibits cell growth. The frequent loss of hepaCAM in human cancers and its inhibitory effects on cell growth fulfill two of the most important criteria to define tumor suppressors.

The present study aims to further investigate the characteristics of hepaCAM, mainly focusing on two subjects: 1) the connection between hepaCAM and the integrin-dependent signaling, and 2) the proteolytic cleavage of hepaCAM.

Besides the suppressive effects on cell growth, hepaCAM promotes cell-ECM adhesion and cell motility. Since integrins play a central role in cell-ECM interactions,
it would be interesting to investigate the relationship between hepaCAM and integrins. The first study demonstrates that when re-expressed in MCF7 cells, hepaCAM accelerates the initial cell attachment and spreading on a fibronectin substrate. Both the velocity and directionality of cell migration are also enhanced. In addition, hepaCAM is found to be physically associated with integrin β1, the most highly expressed integrin in MCF7 cells; moreover, hepaCAM up-regulates the protein expression of integrin β1 as well as its active form. Interestingly, hepaCAM regulates the assembly of the focal contacts time dependently. The unique regulation allows the cells to adhere to the ECM quickly and adapts to a migration mode in an efficient way. Furthermore, hepaCAM alters the activity of Rho family GTPases. At last, the altered signaling mediated by hepaCAM results in an altered organization of the actin cytoskeleton, which supports a migratory phenotype. These results indicate that although possessing the properties of a tumor suppressor, given an environment that favors cell migration, hepaCAM may work in concert with integrin signaling pathway to contribute to a more migratory phenotype in cancer.

Proteolytic cleavage is a common process for many membrane-anchored proteins. In the second study, a proteolytic cleavage of hepaCAM is identified. This cleavage generates a 25-kD product containing mainly the cytoplasmic domain of hepaCAM. The phorbol ester phorbol 12-myristate 13-acetate (PMA) has no effect on hepaCAM cleavage. However, the cleavage is promoted by the Ca^{2+} ionophore ionomycin. In addition, inhibitors of proteasome and cysteine proteases strongly suppress the cleavage of hepaCAM, indicating the involvement of proteasome, calpain-1 and cathepsin B. Furthermore, the cytoplasmic truncated mutant of hepaCAM fails to
promote cell-ECM adhesion and migration, and loses the inhibitory effect on cell growth, suggesting a regulatory role of the cleavage in hepaCAM functions.

In conclusion, the first study uncovers a novel connection between hepaCAM and the integrin-dependent signaling, providing the molecular basis for the hepaCAM-mediated cell-ECM adhesion and migration. The second study explores the mechanisms that are involved in hepaCAM cleavage and sheds light on the possibility of hepaCAM cleavage functioning as a regulatory switch. Further explorations are needed to better understand hepaCAM and its roles in physiological and pathological processes.
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ABBREVIATIONS

ADAM – a disintegrin and metalloprotease

AJ – adherens junction

ANOVA – analysis of variance

BLAST – basic local alignment search tool

bp – base pair

BSA – bovine serum albumin

CA074-Me – CA074 methyl ester

CEACAM – carcinoembryonic antigen cell adhesion molecule

CMAC – cell-matrix adhesion complex

CNS – central nervous system

DAPT – N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DGC – dystrophin-glycoprotein complex

DMEM – Dulbecco’s modified Eagle’s medium

DMSO – dimethyl sulfoxide

DN – double negative

DSP - dithiobis(succinimidylpropionate)

DTT – dithiothreitol
DTSSP - 3,3′-Dithiobis(sulfosuccinimidylpropionate)

ECM – extracellular matrix

EDTA - Ethylenediaminetetraacetic acid

FA – focal adhesion

FAK – focal adhesion kinase

FBS – fetal bovine serum

FERM – 4.1 ezrin/radixin/moesin

FX – focal complex

GAP – GTPase activation protein

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GDP – guanosin diphosphate

GEF – guanine nucleotide exchange factor

GTP – guanosin triphosphate

HCC – hepatocellular carcinoma

HD – hemidesmosomes

HS – heparin sulfate

IgCAM – immunoglobulin-like cell adhesion molecule

JAM – junctional adhesion molecule

kb – kilobase
kDa – kilodalton

LB – Luria broth

LIMK – LIM domain kinase

MG132 – N-(benzyloxy carbonyl)leucinyl-leucinyl-leucinal

MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OD – optical density

ORF – open reading frame

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PCK – protein kinase C

PDZ – PSD-95/Dig/ZO-1

PG – preteoglycan

PMA – phorbol 12-myristate 13-acetate

PVDF – polyvinylidene

RIPA – radioimmunoprecipitation assay

SDS – dodecyl sulfate

TBS – tris buffered saline

TBS/T – tris buffered saline/Tween 20

TJ – tight junction
TRITC – tetramethylrhodamine isothiocyanate

TSLC1 – tumor suppressor in lung cancer 1

WT – wild-type

Z-FF-FMK – Z-phe-phe-fluoromethylketone

ZO – zona occluden
CHAPTER 1 INTRODUCTION

1.1 Cell adhesion molecules

Cell adhesion is a dynamic process essential for the normal development and maintenance of tissues and organs in metazoans (multicellular organisms). Accumulating evidences have revealed that cell adhesion is crucial not only for the cellular architecture but also for various biological processes including cell migration, proliferation and survival (Edelman, 1986). Cell-cell and cell-extracellular matrix (ECM) interactions are mediated by a large and complex number of cell adhesion molecules (CAMs) expressed on the cell surface that interact with one another in a spatially and temporally regulated manner.

It has been more than half a century since the first CAMs were identified. The observation of the ability of cells in completely disrupted chick embryo tissues to reassemble and reform the original structure suggested the existence of adhesion molecules on the cell membrane (Moscona, 1952). Since the pioneering work of Moscona, Townes and Holtfreter in the 1950s, significant steps have been made in the identification, characterization and classification of several distinct cell adhesion systems (Moscona, 1952; Townes and Holtfreter, 1955). These systems allow cells to interact dynamically with adjacent cells and the ECM. They are important in the development, morphogenesis, maintenance and regeneration of the structure and organization of organisms (Edelman, 1986; Gumbiner, 1996). They also play important roles in pathological conditions such as tumor formation and metastasis (Takeichi, 1991).
To date, hundreds of CAMs have been identified and characterized. According to their structural and functional features, they are classified into five major families: immunoglobulin-like CAMs (IgCAMs), integrins, selectins, cadherins and heparin sulfate proteoglycans (HSPG).

1.1.1 The immunoglobulin-like CAMs

IgCAMs belong to the immunoglobulin superfamily, a widespread and complex protein family of more than 100 members. IgCAMs function as cell adhesion and signaling receptors that transduce extracellular signals from neighbouring cells or the ECM to the intracellular signaling machinery.

Structurally, IgCAMs contain one or more Ig-like domains capable of binding to other protein motifs (Brummendorf and Rathjen, 1995). Ig-like domains usually contain two conserved cysteine residues linked by intrachain disulfide bonds. Structural diversity is achieved by post-translational modification of extracellular domains by glycosylation, or by modification of intracellular domains through serine, threonine, or tyrosine phosphorylation (Brummendorf et al., 1998). Additional variants of some IgCAMs are generated by alternative splicing that changes the domain organization.

Certain IgCAMs are expressed in a wide variety of tissues suggesting a general biological role (Brummendorf and Rathjen, 1995) while the others show more restricted expression patterns consistent with tissue-specific functions. Expression of IgCAMs is often regulated during development. The diversity of IgCAMs created by
post-translational modification or alternative splicing may also be subjected to developmental or tissue-specific regulation (Hassel et al., 1997).

Through their extracellular domains, IgCAMs interact with numerous cell surface molecules including other IgCAMs and molecules belonging to different protein families. Some IgCAMs, e.g. the L1-like molecules, NCAM, and axonin-1, display homophilic binding, i.e. the interaction between two identical molecules. However, most IgCAMs interact heterophilically either with non-identical IgCAMs or with structurally unrelated partners. IgCAMs expressed by one cell can bind to interaction partners on membranes of the opposing cells in trans, or bind laterally to molecules on the same membrane plane in cis. The ability of IgCAMs to interact with a large variety of molecules suggests its involvement in a complicated network of molecular interactions. In addition to the diverse and complex extracellular interactions, the cytoplasmic domains of transmembrane IgCAMs also participate in intracellular interactions. Thus IgCAMs may play an important role in cell motility. For example, the cytoplasmic domains of some IgCAMs are able to bind to components of the cortical cytoskeleton like actin and ankyrins (Brummendorf et al., 1998).

IgCAMs also play important roles in signal transductions. Evidences have been provided that IgCAMs are associated with different signaling molecules like receptor and non-receptor tyrosine kinases, receptor protein tyrosine phosphatases (RPTP), and various serine/threonine kinases (Aricescu et al., 2007).
1.1.2 Integrins

Integrins are the major metazoan receptors for cell adhesion to ECM proteins and, in vertebrates, also play important roles in certain cell-cell adhesions. Since the recognition of the integrin receptor family 24 years ago (Hynes, 1987), they have become the best understood cell adhesion receptors. In addition to mediating cell adhesion, integrins make transmembrane connections to the cytoskeleton and activate numerous intracellular signaling pathways. Integrins and their ligands play key roles in development, immune responses, leukocyte traffic, hemostasis, and cancer and are at the heart of many human diseases – genetic, autoimmune, and others. They are the target of effective therapeutic drugs against thrombosis and inflammation, and are receptors for some viruses and bacteria (Hynes, 2002).

Integrins are restricted to the metazoans; no homologs are detected in prokaryotes, plants, or fungi (Whittaker and Hynes, 2002). It is plausible that evolution of integrins was necessary to allow the cell-matrix adhesion intrinsic to metazoans (Hynes, 2002). In human, integrin family is so far known to comprise 8 β and 18 α subunits, assembling into 24 distinct integrin heterodimers. Each of the α and β subunit crosses the membrane once, with most of each polypeptide (>1600 amino acids in total) in the extracellular space and two short cytoplasmic domains (20-50 amino acids). Each of the 24 heterodimers appears to have specific, nonredundant function which is evident from their ligand specificities and distinct phenotypes of knockout mice (Hynes, 2002).
Cell-cell and cell-ECM adhesions are mediated by the binding of integrin extracellular domains to diverse protein ligands; in addition, integrins serve as transmembrane mechanical links from those extracellular contacts to the cytoskeleton inside cells. For all integrins except α6β4, the linkage is to the actin-based microfilament system, which integrins also regulate and modulate. The β4 subunit differs from all the others; its cytoplasmic domain is much longer, about 1000 amino acids long instead of around 50, making connections to intermediate filaments instead of to actin (Hynes, 2002). In part related to the integrin-mediated assembly of cytoskeletal linkages, ligation of integrins also triggers a large variety of signal transduction events that serve to modulate many aspects of cell behavior including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation. These signal transduction pathways are complex, like those emanating from receptors for soluble factors (e.g. G protein-coupled and kinase receptors). In fact, many cellular responses to soluble growth factors, such as EGF, PDGF, LPA, and thrombin, etc., are dependent on the cell’s being adherent to a substrate via integrins. That is the essence of anchorage dependence of cell survival and proliferation and integrins lie at the basis of these phenomena (Schwartz and Assoian, 2001; Frisch and Screaton, 2001).

One important mechanism by which integrin function is regulated is through tight spatial and temporal control of integrin affinity for extracellular ligands. This is achieved by rapid, reversible changes in the conformation of the extracellular domains of the integrin heterodimer, so-called integrin activation (Woodside et al., 2001). Despite significant recent advances, the exact nature of the conformational changes leading to integrin activation remains controversial. Additional affinity-independent
mechanisms, such as integrin clustering, lateral diffusion of receptors, interactions with and reorganization of the cytoskeleton, and changes in integrin expression patterns also contribute to the control of integrin-mediated adhesion (Laudanna et al., 2002; van Kooyk and Figdor, 2000; Hogg et al., 2002). Affinity-dependent and – independent mechanisms are not mutually exclusive and can act in concert; indeed, integrin activation and clustering may be mechanistically linked such that activation, in combination with ligand occupancy, also stimulates clustering (Erb et al., 1997; Li et al., 2002). It has been widely accepted that the membrane-proximal regions of the short α and β integrin cytoplasmic tails are well conserved and play crucial roles in integrin activation, probably by interacting with one another to stabilize an inactive conformation. The more distal region of the β tails regulate activation through interactions with signaling proteins that might disrupt the membrane-proximal interaction, whereas membrane-distal α sequences regulate β tail conformation and association with activator proteins in a cell-type-specific manner (Ylanne et al., 1993). Several studies indicate that talin, a major cytoskeletal actin-binding protein that binds to integrin tails and colocalize with activated integrins (Critchley 2000), plays a crucial role in integrin activation (Tadokoro et al., 2003). Studies also showed that at least two other β tail-binding proteins, β3-endoexin (Kashiwagi et al., 1997) and cytohesin (Kolanus et al., 1996), and one α tail-binding protein, calcium- and integrin-binding protein (CIB) (Tsuboi, 2002), might also directly activate integrins.

1.1.3 Cadherins

Cadherins (named for “calcium-dependent adhesion”) are a superfamily of type-1 transmembrane proteins important for cell-cell adhesion. Their functions are
dependent on calcium ions, hence their name. The cadherin superfamily includes classical cadherins, protocadherins, desmogleins, desmocollins, etc (Hulpiau and van Roy, 2009). The classical cadherins are single-span transmembrane proteins located primarily within adherens junctions, mediating calcium-dependent cell-cell adhesion through their five extracellular calcium binding repeats. E-cadherin and N-cadherin have been the best characterized and studied. Their intracellular regions link them with their cytoplasmic partners β-catenin or plakoglobin and consequently to α-catenin and the actin filament network (Yap et al., 1997a, b). The desmosomal cadherins are the transmembrane protein components of desmosomes, which are sites of cell-cell adhesion present particularly in tissues subjected to mechanical strains (e.g. epithelia, particularly epidermis, and the myocardium). There are two subfamilies of desmosomal cadherins, the desmocollin and desmoglein proteins, and each possesses three subtypes which are expressed in a cell-type and differentiation-specific manner (King et al., 1997).

### 1.1.4 Selectins

Selectins are single-chain transmembrane glycoproteins that share similar properties to C-type lectins due to a related amino terminus and calcium dependent binding. Selectins bind to sugar moieties and so are considered to be a type of lectin, cell adhesion proteins that bind sugar polymers. Selectins were firstly cloned in 1989. The main types include E-selectin, P-selectin, and L-selectin (Kansas, 1996).

L-selectin is expressed on leukocytes (Kansas et al., 1985; Lewinsohn et al., 1987). It is rapidly lost from the surface of normal leukocytes in response to a variety of stimuli.
Soluble L-selectin can be detected in the serum of normal healthy individuals at high concentrations (Schleiffenbaum et al., 1992). The mechanism of shedding is not completely understood, but is clearly the result of proteolytic cleavage at a site just outside the plasma membrane. E-selectin expression is limited to endothelium, and principally to endothelium in response to inflammatory stimuli (Bevilacqua MP, et al., 1989). The loss of E-selectin from the surface of activated endothelial cells is caused by several factors. First, the half-life of E-selectin mRNA is short. In addition, E-selectin is rapidly internalized and degraded in lysosomes (Subramaniam et al., 1993). The combination of these processes collectively ensures that the expression of E-selectin at the surface of cytokine-stimulated endothelium is transient. P-selectin expression on both endothelium and platelets is also inducible. However, P-selectin is stored preformed in the α-granules and Weibel-Palade bodies of platelets and endothelium (McEver et al., 1989). P-selectin is rapidly expressed at the cell surface as a result of fusion of these granules with the plasma membrane. The sorting of P-selectin into these granules is controlled by sequences within the cytoplasmic tail. P-selectin expressed at the cell surface is rapidly internalized, which accounts for its transient appearance at the surface of activated endothelium and platelets, and this activity also maps to the cytoplasmic tail (Green et al., 1994).

1.1.5 Heparan sulfate proteoglycan

Heparin sulfate (HS) is a linear polysaccharide found in all animal tissues. It occurs as a proteoglycan (PG) in which two or three HS chains are attached in close proximity to cell surface or ECM proteins. HS binds to a variety of protein ligands and regulates a wide variety of biological activities, including developmental processes,
angiogenesis, blood coagulation and tumour metastasis. The major cell membrane HSPGs are the transmembrane syndecans (Rapraeger, 1983) and the glycosylphosphatidylinositol anchored glypicans. Other minor forms of membrane HSPGs include betaglycan and the V-3 isoform of CD44 present on keratinocytes and activated monocytes. Cell surface HS sequesters secreted soluble ligands and modulates their activity. As coreceptors, HSPGs modulate ligand-receptor encounters that can activate and inhibit cell proliferation, motility, and differentiation. As receptors, HSPGs regulate internalization and clearance of their ligands via both clathrin-coated pits and membrane lipid rafts associated with caveolae.

1.2 Proteolytic cleavage of CAMs

Of membrane-anchored proteins, approximately 1% undergo regulated proteolytic cleavage near the plasma membrane, resulting in truncated extracellular or intracellular segments in a process known as shedding (Hooper et al., 1997; Werb 1997). These functionally diverse proteins include CAMs, cytokines, growth factors, their receptors and enzymes. Shedding of receptors or coreceptors, like syndecans, can produce agonists or antagonists that regulate their ligands’ activities, render cells less responsive to their ligands, or potentially generate an active fragment that remains membrane-associated or becomes intracellular. Other examples include the cleavage of CD44, which plays a critical role in cancer cell migration and metastasis and various physiological events (Okamoto et al., 2001); proteolysis of E-cadherin regulates epithelial cell-cell adhesion, migration, and β-catenin translocation (Maretzky et al., 2005a); cleavage of the neural adhesion molecule L1 regulates neural cell adhesion, migration and neurite outgrowth (Maretzky et al., 2005b).
The shedding process of CAMs is usually differentially regulated by multiple pathways. It has been shown that CD44 cleavage is under the control of protein kinase C (PKC), Ca$^{2+}$ influx, Rho GTPase proteins (Okamoto et al., 1999), membrane-type 1 matrix metalloprotease (Kajita et al., 2001), and γ-secretase (Pelletier et al., 2006). E-cadherin cleavage is promoted by Ca$^{2+}$ influx (Ito et al., 1999) and regulated by γ-secretase (Marambaud et al., 2002) and a disintegrin and metalloprotease 10 (ADAM10) (Maretzky et al., 2005a). L1 is processed by ADAM10, ADAM17, and γ-secretase (Maretzky et al., 2005b).

1.3 Types of cell adhesion

1.3.1 Cell-cell adhesions

1.3.1.1 Gap junctions

A gap junction or nexus is a specialized intercellular connection between cells of multi-cellular animals (White and Paul, 1999). It directly connects the cytoplasm of two cells, which allows various molecules and ions to pass freely between cells (Lampe et al., 2004). One gap junction channel is composed of two connexons (or hemichannels) which connect across the intercellular space (Maeda et al., 2009). At gap junctions, the intercellular space is 4 nm (Maeda et al., 2009) and unit connexons in the membrane of each cell are lined up with one another (Perkins et al., 1998). There are four levels of organization of a gap junction: 1) one connexin protein is composed of four transmembrane domains; 2) six connexins form one connexon
(hemichannel); 3) two hemichannels that joined together across a cell membrane comprise a gap junction channel. A gap junction channel is homotypic when it is formed by two identical connexons and heterotypic when it is composed of one homomeric and one heteromeric connexon. Two heteromeric connexons also form heteromeric gap junction channel; 4) finally, several gap junction channels (can be hundreds) assemble into a macromolecular complex called a gap junction. Gap junctions allow direct electrical and chemical communication between cells. Moreover, molecules smaller than 1000 Daltons are generally allowed to pass through. Large biomolecules, e.g. nucleic acid and protein, are precluded from cytoplasmic transfer between cells. These properties ensure that molecules and current passing through the gap junctions do not leak into the intercellular space.

1.3.1.2 Adherens junctions

Adherens junctions (AJs) link cell membrane and cytoskeletal components at discrete contact regions. The most well known example of AJ is the zonular adherens junction, or zonular adherens of polarized epithelial cells. While the zonula adherens is continuous or “belt-like” in most epithelia, AJs are often “spot-like”. Spot-like junctions may favor anchoring, while a zonular “belt-like” junction enables coordination of epithelial sheet movements.

AJs consist of two basic adhesive units: the cadherin/catenin and nectin/afadin complexes. The cadherin cytoplasmic domain mediates key structural and signaling activities required for adhesion through its association with three distinct proteins
known as catenins (Ozawa et al., 1989). β-catenin is an arm-repeat protein (Coates et al., 2003) whose direct binding to cadherin is crucial for full adhesive function. α-catenin is a vinculin homologue that can bind F-actin in vitro (Rimm et al., 1995), and is crucial for actin polymerization at or near AJs in vivo. Cadherins also associate with p120<sup>ctn</sup>, which belongs to a subfamily of armadillo proteins (Alema and Salvatore, 2007). Nectin is a member of the IgG superfamily of calcium-independent adhesion molecules. The nectin subfamily contains an extracellular domain comprised of three IgG-like loops, and a cytoplasmic domain, which contains a C-terminal PDZ-binding motif in most variants (Irie et al., 2004). Nectins form lateral homodimers that can engage in both homophilic and heterophilic adhesion with other nectins or nectin-like receptors. The cytoplasmic domain of nectin interacts with an actin-binding protein known as AF6/afadin (Zhadanov et al., 1999), thereby providing an alternate way to couple AJs to actin. Thus, like the cadherin/catenin adhesive unit, the nectin-afadin complex contains components that can mediate intercellular adhesion and actin association. Besides mediating the basic adhesiveness of cells, AJs also serve functions such as contact mediated growth inhibition, invaginations, and establishment of apical-basolateral polarity (Nejsum and Nelson, 2007).

1.3.1.3 Desmosomes

The desmosomes are adhesive intercellular junctions that are crucial to tissues that experience mechanical stress, such as the myocardium, bladder, gastrointestinal mucosa, and skin (Holthofer et al., 2007). Desmogleins and desmocollins (Garrod and Chidgey, 2008), members of the cadherin superfamily, mediate adhesion at desmosomes. The desmogleins and desmocollins both contain four extracellular
cadherin homology repeats and a fifth domain termed the extracellular anchor. Desmosomes show Ca\(^{2+}\)-dependent assembly and adhesion. Classical cadherins typically show homophilic interactions, which support cell-cell adhesion and tissue patterning. Most studies to date suggest that both desmocollins and desmogleins are required for strong cell-cell interactions (Marcozzi et al., 1998; Getsios et al., 2004). However, it remains unclear whether homophilic or heterophilic interactions are primarily responsible for desmosome adhesion. In the desmosome, the cytoplasmic associations with the cadherins are partly mediated by the armadillo family proteins, plakoglobin, and the plakophilins. The armadillo proteins include β-catenin, plakoglobin (γ-catenin), p120-catenin, p0071, ARVC, δ-catenin, and the plakophilins 1–3 (Hatzfeld, 2005; Hatzfeld, 2007). The desmosomal cadherins show complex developmental and differentiation patterns of expression (Holthofer et al., 2007). They play important roles in epithelial proliferation and differentiation, and in the robust cell-cell adhesion that is required for tissue integrity. Desmosomal cadherins are implicated in human diseases, particularly in disorders affecting the heart and the skin. In addition to inherited disorders, desmosomal cadherins are also targets of autoimmune disease such as pemphigus.

1.3.1.4 Tight junctions

Tight junctions (TJs) regulate the passage of ions and molecules through the paracellular pathway in epithelial and endothelial cells (Lopez-Bayghen et al., 2005). These structures are located at the limit between the apical membrane facing the lumen and the basolateral surface in contact with the interior of the organism. The barrier function of TJs allows this structure to regulate by size and charge the transit
of ions and molecules through the paracellular route (Anderson and Cereijido, 2001). Besides functioning as a barrier, TJs work as a fence that limits the free movement of lipids and proteins within the plasma membrane between the apical and the basal surface (Mandel et al., 1993).

TJs are multiprotein complexes composed of integral proteins that associate with cytoplasmic plaque proteins. The former mediate cell-cell adhesion, while the latter function as bridge between the TJ and the actin cytoskeleton (Schneeberger and Lynch, 2004). The main molecular constituents of TJ include zona occludens (ZO) proteins, claudins and occludin. ZOs are peripherally associated membrane proteins. These proteins interact together and anchor membrane proteins like claudins, occluding and juncional adhesion molecules (JAMs) to the actin cytoskeleton. ZO proteins are scaffolds that establish numerous protein-protein interactions that cluster at the TJ diverse kinase, phosphatases, small G proteins and nuclear and transcription factors (Lopez-Bayghen et al., 2005). Claudins constitute a family of more than 20 proteins with four transmembrane domains (Morita et al., 1999). They have two extracellular loops that display variability in the distribution and number of charged residues. This feature is crucial as it determines the paracellular ionic selectivity of the TJ. Occludin is present in the filaments that constitute TJs and its overexpression or mutation severely affect transepithelial electrical resistance (McCarthy et al., 1996), yet occludin knock-out mice are viable and exhibit TJs with an apparent normal morphology (Saitou et al., 2000).
1.3.2 Cell-extracellular matrix adhesion

1.3.2.1 Extracellular matrix

The ECM is a complex structural entity surrounding and supporting cells that are found within mammalian tissues. The ECM is often referred to as the connective tissue. The ECM is composed of 4 major classes of molecules: 1) structural proteins, e.g. collagen and elastin; 2) specialized proteins, e.g. fibrillin, fibronectin, and laminin; 3) PGs: these are composed of a protein core to which is attached long chains of repeating disaccharide units termed of glycosaminoglycans forming extremely complex high molecular weight components of the ECM; 4) non-PG polysaccharide, e.g. hyaluronan.

1.3.2.2 Cell-matrix adhesion complexes

Cell-matrix adhesion complexes (CMACs) mechanically link the cell to the ECM (Delon and Brown, 2007). CMACs form upon integrin ligation to the ECM and subsequent integrin clustering. This rapidly induces the recruitment of an array of CMAC signaling and adaptor proteins, forming large intracellular protein complexes bound both to clustered integrin cytoplasmic tails and to actin microfilaments. CMACs fulfill a variety of functions in the cell. Besides physically attaching cells to the ECM, CMACs also play important roles in the creation, mediation and sensing of tension (Geiger and Bershadsky, 2001). In addition, CMACs are influential signaling hubs that detect, coordinate, transmit, adapt to and generate various signals regulating virtually all core cellular functions.
CMACs differ significantly in features such as size, shape, location, components, dynamics and linkage to F-actin. Based on this, CMACs have been divided into a number of different categories (Zamir et al., 1999). Most of these CMAC categories represent different adhesion maturation states, including: focal points or nascent adhesions - small, often newly formed CMACs in the cellular periphery that link to an F-actin meshwork; focal complexes (FXs) - mid-sized stationary contact sites linked to the cortical actin and/or the actin meshwork within lamellipodia; focal adhesions (FAs) – large adhesion sites that elongate along the axis of force application by actin stress fibers to which they connect; fibrillar adhesions – elongated adhesions that connect microfilament stress fibers with extracellular fibronectin fibers; and podosomes – invasive ring structures composed of adhesion machinery and filamentous actin.

The main components of CMACs include integrins, adaptor and signaling proteins, and microfilament structures. CMAC components function either as adaptors or as signaling molecules, or both. Adaptors may form physical links between, and/or may act as scaffolds for signaling molecules. For example talin, which serves as a physical link between integrins and F-actin, is a scaffold coordinating other CMAC components such as vinculin and PIPKιγ (Ling et al, 2006), and may be involved in transduction of tension signaling (Critchley, 2004). Likewise, while focal adhesion kinase (FAK) displays inherent kinase activity, the main functions of FAK appear to be connected to its capacity as a signaling scaffold (Schlaepfer, 1999). Other important examples of CMAC components include paxillin, a highly regulated and dynamic scaffold protein; kindlins, which may associate with integrins and form a link to actin via migfillin and filamin (Wu, 2005), and act as activators of integrins. Pinch, which forms a ternary complex with integrin-linked kinase and parvin and
functions to regulate gene transcription and cell-cell adhesion (Legate et al., 2006); zyxin, which incorporates into CMACs in a tension-dependent manner (Zaidel-Bar et al., 2004); p130Cas, which may serve as a transducer of both chemical and mechanical signaling (Defilippi et al., 2006); c-Src, a key modifier of many CMAC scaffolds, such as paxillin and FAK, allowing them to transmit signaling further (Frame et al., 2002); and various regulators of small GTPase signaling, including the α- and β-PIX guanine nucleotide exchange factors (GEFs) (Rosenberger and Kutsche, 2006). The actin microfilament system structure and dynamics are to a large extent regulated by CMACs and their signaling, and the tension created jointly by the CMACs and microfilaments affects both structures. Different dynamic microfilament structures are physically linked to CMACs, and each is connected to distinct types of CMACs. These microfilament structures include lamellipodia, filopodia, stress fibers and podosomes (Spinardi and Marchisio, 2006).

In general, cell-ECM adhesion promotes integrin clustering and recruitment of integrin-associated proteins to integrin-rich adhesion sites (Fig. 1-1). One important event in this process is cell adhesion-dependent phosphorylation of key focal adhesion proteins such as FAK (Parsons, 2003). Phosphorylation of FAK at Tyr-397 creates a docking site for the SH2 domain of Src family kinases. Binding of Src to FAK phosphor-Tyr-397 site releases an autoinhibitory interaction and consequently activates Src. Activated FAK/Src complex in turn phosphorylates components of FAs including FAK, paxillin and p130Cas, resulting in recruitment of additional signaling intermediates and activation of downstream signaling pathways. A second important event is the recruitment of key protein complexes such as the pinch-ILK-parvin ternary complexes (Wu, 2004) to cell-ECM contacts. The complexes enhance the
Figure 1-1 Integrin pathway. Upon ligation to the ECM, integrins are activated and clustered. An array of adaptor proteins are rapidly recruited, forming large intracellular protein complex bound both to clustered integrin cytoplasmic tails and to actin microfilaments. One important event in this process is phosphorylation of key focal adhesion proteins such as FAK. Phosphorylation creates docking sites for other proteins such as Src family kinases. The activated FAK/Src complex in turn phosphorylates paxillin and p130Cas, resulting in recruitment and activation of additional signaling intermediates including the Rho family GTPases, leading to various cellular changes.

Figure adapted from https://www.qiagen.com/geneglobe/pathwayview.aspx?pathwayID=254
physical connection between integrins and the actin cytoskeleton. The third important event is the changes of the conformation of certain FA proteins in response to physical forces exerted on FA. For example it has been shown that the conformation and tyrosine phosphorylation of p130Cas are changed in vitro as well as in cells in response to mechanical stretches (Sawada et al., 2006).

1.3.2.3 Hemidesmosomes

Hemidesmosomes (HD) are rivet-like structures on the inner basal surface of keratinocytes in the epidermis of skin. They are similar in form to desmosomes when visualized by electron microscopy (Nguyen et al., 2006). While desmosomes link two cells together, HDs attach one cell to the ECM. Rather than using cadherins, HDs use integrins. The HD comprises two rivet-like plaques (the inner and outer plaques); together with the anchoring fibrils and anchoring filaments these are collectively termed the HD-stable adhesion complex or HD-anchoring filament complex. Together, the HD-anchoring filament complex forms a continuous structural link between the basal keratinocyte keratin intermediate filaments and the underlying basement membrane zone and dermal components. An example configuration of an HD consists of cytosolic keratin, non-covalently bonded to a cytosolic plectin plaque, which is bonded to a single-pass transmembrane adhesion molecule such as the α6β4 integrin (Nguyen et al., 2006).
Dystroglycan was originally isolated from skeletal muscle as an integral membrane component of the dystrophin-glycoprotein complex (DGC) (Ervasti et al., 1990). In vertebrates dystroglycan is composed of α- and β-subunits encoded by a single gene and cleaved into two proteins by posttranslational processing (Ibraghimov-Beskrovnaya et al., 1992). At the sarcolemma, β-dystroglycan binds intracellularly to dystrophin, which binds to the actin cytoskeleton, and extracellularly to α-dystroglycan, a highly glycosylated peripheral membrane protein. It completes the link from the cytoskeleton to the basal lamina by binding to ECM proteins containing LamG domains, such as laminin, neurexin, agrin, and perlecan. In addition to dystroglycan and dystrophin, the DGC in muscle cells contains the sarcoglycan complex composed of five sarcoglycan proteins (α, β, γ, δ, ζ) and sarcospan (Wheeler and McNally, 2003). Via dystrophin, the sarcolemmal DGC interacts with a pair of syntrophins (α1 and β1) and α-dystrobrevin within the cytosol (Peters et al., 1997; Sadoulet-Puccio et al., 1997). Though the exact function of the DGC is not entirely understood, it is thought to contribute to the structural stability of the muscle cell membrane during cycles of contraction and relaxation, thereby protecting the muscle from stress-induced membrane damage (Campbell, 1995). In humans, mutations in dystrophin cause Duchenne and Becker muscular dystrophy; mutations in sarcoglycans cause limb-girdle muscular dystrophy; and mutations in laminin α2 cause congenital muscular dystrophy (Mathews, 2003).
1.4 Cell migration

1.4.1 The migration cycle

Cell migration can be conceptualized as a cyclic process (Lauffenburger and Horwitz, 1996). The initial response of a cell to a migration-promoting agent is to polarize and extend protrusions in the direction of migration. These protrusions can be large, broad lamellipodia or sipe-like filopodia, are usually driven by actin polymerization, and are stabilized by adhering to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. These adhesions serve as traction sites for migration as the cell moves forward over them, and they are disassembled at the cell rear, allowing it to detach (Ridley et al., 2003).

A cell’s migratory behavior depends on its environment. Somatic cells migrating in vivo, for example, show large single protrusions and highly directed migration, in contrast to the multiple small protrusions they display one planar substrates; cancer cells can modify their morphology and nature of migration in response to environmental changes (Knight et al., 2000; Friedl and Wolf, 2003).

1.4.2 The protrusive machinery

Actin filaments are intrinsically polarized with fast-growing “barbed” ends and slow-growing “pointed” ends, and this inherent polarity is used to drive membrane protrusion. However, the organization of filaments depends on the type of protrusion: in lamellipodia, actin filaments form a branching “dendritic” network, whereas in filopodia they are organized into long parallel bundles. Actin polymerization in
lamellipodia is mediated by the Arp2/3 complex, which binds to the sides or tip of a pre-existing actin filament and induces the formation of a new daughter filament that branches off the mother filament (Welch and Mullins, 2000; Pollard and Borisy, 2003). Several actin-binding proteins regulate the rate and organization of actin polymerization in protrusions by affecting the pool of available monomers and free end (Pollard and Borisy, 2003; dos Remedios et al., 2003). For example, profilin prevents self-nucleation by binding to actin monomers and also serves to selectively target monomers to barbed ends. Filament elongation is terminated by capping proteins, thereby restricting polymerization to new filaments close to the plasma membrane. In addition, disassembly of older filaments, which is needed to generate actin monomers for polymerization at the front end, is assisted by proteins of the ADF/cofilin family, which sever filaments and promote actin dissociation from the pointed end.

Filopodial protrusion is thought to occur by a filament treadmilling mechanism, in which actin filaments within a bundle elongate at their barbed ends and release actin monomers from their pointed ends (Welch and Mullins, 2000). The long and unbranched filament organization is consistent with assembly occurring by elongation rather than by branched nucleation. Many proteins are enriched at filopodial tips, including Ena/VASP proteins, which bind barbed ends of actin filaments and antagonize both capping and branching, thereby allowing continuous elongation of filaments and fascin, which bundles actin filaments and might thereby generate the stiffness needed to allow efficient pushing of the plasma membrane in filopodia (Welch and Mullins, 2000).
The supramolecular design of lamellipodia and filopodia endows them with the capacity to perform distinct functions. Biophysical considerations suggest that the dendritic organization of lamellipodia provides a tight brush-like structure that is able to push along a broad length of plasma membrane (Pollard and Borisy, 2003). Through localized activation of the Arp2/3 complex, the lamellipodium could be induced to grow in a particular direction, providing the basis for directional migration. In contrast, filopodia, with their parallel bundle organization, are particularly well designed to serve as sensors and to explore the local environment, although they are not essential for chemotaxis.

1.4.3 Rho family GTPases

The Rho family small guanosine triphosphate (GTP)-binding proteins (GTPases) are pivotal regulators of actin and adhesion organization and control the formation of lamellipodia and filopodia. They are conformationally regulated by the binding of GTP and guanosin diphosphate (GDP): when bound to GTP, they are active and interact with their downstream target proteins, which include protein kinases, lipid-modifying enzymes, and activators of the Arp2/3 complex (Etienne-Manneville and Hall, 2002). Rho GTPases are activated by GEFs and inactivated by GTPase activation proteins (GAPs) (Moon and Zheng, 2003; Zheng, 2001). Once activated, Rho GTPases interact with cellular target proteins (effectors) to generate a downstream response. To date, more than 40 effectors, 50 GEFs and 40 GAPs have been described for the mammalian Rho family.
Rac and Cdc42 are both required at the front of migrating cells. The primary role of Rac is to generate a protrusive force through the localized polymerization of actin. Cdc42 induces actin polymerization to generate filopodia often seen at the front of migrating cells (Nobes and Hall, 1995). Cdc42 also plays a crucial role in controlling the direction of migration. The Ser/Thr kinase p65PAK is commonly activated upon either Rac or Cdc42 activation and is believed to play an important role in regulating actin dynamics and cell adhesion during migration. p65PAK regulates FA turnover, with the help of PIX and GIT1, and phosphorylates and activates LIM kinase, which in turn phosphorylates and inactivates cofilin (Arber et al., 1998). Cdc42 also activates WASp and N-WASp directly (Rohatgi et al., 1999, 2000). Rac activates the Scar/WAVE family indirectly and this involves an Nck-adaptor complex (Eden et al., 2002).

Rho activity in migrating cells is associated with FA assembly and cell contractility and is responsible for cell body contraction and rear end retraction. One important Rho target involved in stimulating actin:myosin filament assembly and therefore contractility is the Ser/Thr kinase p160ROCK. p160ROCK, like p65PAK, can phosphorylate and activate LIMK, which in turn phosphorylates and inactivates cofilin leading to stabilization of actin filaments within actin:myosin filament bundles (Maekawa et al., 1999). p160ROCK interacts with and phosphorylates the myosin binding subunit of myosin light chain phosphatase and thereby inactivates it (Kawano et al., 1999). This leads to increased levels of myosin phosphorylation, which then can cross-link actin filaments and generate contractile force. At the rear of a migrating cell, this promotes movement of the cell body and facilitates detachment of the cell rear. Clearly, Rho activity at the front of a migrating cell is incompatible with membrane
protrusion and hence its activity must be inhibited at the leading edge. One such mechanism is through Rac. Expression of activated Rac has been shown to inhibit Rho function in many cell types, ranging from fibroblasts to neurons (Sander et al., 1999).

The activity of Rho-family GTPases is highly regulated in space and in time. Most of what is summarized above has been derived from techniques that do not resolve these dimensions. Recently, new imaging technologies allowing spatio-temporal resolution has led to insights that significantly extend the classic model (Pertz, 2010). First, Rho GTPase signaling dynamics occur on micrometer length scales and subminute timescales. Second, multiple subcellular pools of one given Rho GTPase can operate simultaneously in time and space to regulate a wide variety of morphogenetic events. Third, complex spatio-temporal signaling programs that involve precise crosstalk between multiple Rho GTPase signaling modules regulate specific morphogenetic events (Pertz, 2010).

1.5 hepaCAM

1.5.1 Identification of hepaCAM

The identification of hepaCAM was derived from the sequence analysis of another gene, \textit{HEPN1}. Previously, gene \textit{HEPN1} was identified by suppression subtractive hybridization (Moh et al., 2003) in a study aiming to identify altered gene expression in human hepatocellular carcinoma (HCC). \textit{HEPN1} was significantly suppressed in HCC samples, HCC cell lines and cell lines derived from diverse human cancers. Gene \textit{HEPN1} maps to chromosome 11q24.2; the protein product consists of 88 amino
acids with a molecular weight of about 10 kD. The distribution of HEPN1 protein is predominantly intracellular. Functionally, HEPN1 suppresses cell growth and induces apoptosis (Moh et al., 2003).

A BLAST search with the HEPN1 sequence revealed an uncharacterized mRNA sequence of 2465 bp in the database (GenBank AL834419). Interestingly, this sequence contained the entire antisense strand of HEPN1 in its 3’-noncoding region and is deficient in the 5’-noncoding region. Using the gene specific primer at the 5’-end of HEPN1 antisense strand and the adaptor primer, a new gene was identified from a human normal liver cDNA library. This new gene was later designated hepaCAM (Moh et al., 2005a).

1.5.2 Sequence and structural characteristics of hepaCAM

Gene hepaCAM is mapped to the minus strand of the human chromosome 11q24.2 and contains 7 exons ranging in sizes from 71 to 2252 bp (Moh et al., 2005a). The start codon is located in exon 1 and the stop codon is positioned in exon 7. Intron sizes vary greatly from the longest intron 1 (10,851 bp) to the shortest intron 5 (285 bp).

The full-length cDNA of hepaCAM is 3244 bp long. It contains a 5’ UTR of 44 bp, a deduced coding region of 1251 bp with ATG as start codon and TGA as stop codon, and a 3’ UTR of 1949 bp. An in-frame termination codon TAG is present at 24 nucleotides upstream of the putative initiating methionine, indicating the completion of the ORF. A Kozak sequence (AAAATGA) surrounding the start codon as well as a
Figure 1-2 Identification of *hepaCAM* based on the sequence analysis of *HEPN1*. An uncharacterized mRNA sequence of 2465 bp (AL834419) was identified using a BLAST search with *HEPN1*. This sequence (a partial ORF) contains the entire antisense strand of *HEPN1* in its 3’-noncoding region and is deficient in the 5’-noncoding region. Based on this sequence, a new gene was isolated from a human normal liver cDNA library and designated *hepaCAM*. Gene *hepaCAM* is mapped to human chromosome 11q24 and its genomic DNA sequence contains 7 exons.
polyadenylation sequence (AATAAA), which signals for poly-A addition, are identified in the sequence. \textit{hepaCAM} sequence does not show significant similarity with any known genes.

The protein product of \textit{hepaCAM} is a type I integral transmembrane immunoglobulin (Ig)-like cell adhesion molecule of 416 amino acids (Moh et al., 2005a). hepaCAM protein contains an extracellular domain comprising a signal peptide (residues 1-33) and two Ig-like loops (residues 40-142; 159-224), a transmembrane segment (residues 241-263), and a cytoplasmic tail (residues 264-416). hepaCAM displays structural similarities to Ig-like CAMs like JAM1, CAR and ESAM (Moh et al., 2005a). Typically, they contain an extracellular domain with two Ig-like domains (V and C2 domains), a transmembrane segment, and a cytoplasmic tail. Two cysteine residues flanking the C2 Ig domain of hepaCAM contribute to the formation of intrachain disulfide-linked loop (Fig. 1-3).

1.5.3 Expression of \textit{hepaCAM}

Gene \textit{hepaCAM} is widely expressed in normal human tissues including liver, brain, breast, muscle, lung, retina, uterus, etc. (Moh et al., 2008) but significantly downregulated in HCC tissues and suppressed in tissue samples of human tumors of kidney, breast, prostate, uterus, ovary, colon, lung, stomach and rectum (Moh et al., 2005a, 2008).
1.5.4 Subcellular localization of hepaCAM

The subcellular localization of hepaCAM is cell density-dependent (Moh et al., 2005a, b). When the cells are well-spread, hepaCAM protein is localized to punctuate structures in the perinuclear area, cytoplasm, and at the tip of cell surface protrusions that are about to make contact with adjacent cell surfaces, forming zipper-like structures. When the cells are confluent, the hepaCAM is localized to a lesser extent in the perinuclear membrane and cytoplasm, and predominantly on the plasma membrane, particularly at cell-cell boundaries.

1.5.5 Posttranslational modification of hepaCAM

Six N-linked glycosylation sites have been predicted on the extracellular domain of hepaCAM. When expressed in MCF7 cells, the molecular weight of hepaCAM was reduced for ~25 kD after treating with PNGase F, which cleaves N-linked glycans (Moh et al., 2005b). Gaudry et al. (2008) demonstrated that the extracellular domain of hepaCAM is heavily glycosylated in both HEK and CHO cells, with approximately 50% of the molecular mass attributed to glycans. There are significant differences in the glycosylation pattern between hepaCAM expressed in HEK and CHO cells (Gaudry et al., 2008). Since glycosylation can influence the behavior of protein, hepaCAM expressed in different cell lines may possess different properties.

Multiple potential Ser/Thr/Tyr phosphorylation sites are present on the cytoplasmic domain of hepaCAM. A polyclonal antiserum generated by immunizing rabbits with a recombinant cytoplasmic domain of hepaCAM failed to detect the native form of
hepaCAM. However, dephosphorylated form of the protein was detected, indicating that hepaCAM is phosphorylated on the cytoplasmic domain (Moh et al., 2005b).

1.5.6 Dimerization of hepaCAM

Cross-linking studies showed that hepaCAM is able to form homodimer on cell surface through cis-interaction (Moh et al., 2005b). The dimerization is independent of the cytoplasmic domain as a mutant lacking the cytoplasmic segment also forms dimers.

1.5.7 Functional characteristics of hepaCAM

1.5.7.1 Cell growth control by hepaCAM

hepaCAM exhibits inhibitory effect on cell growth. Such effect has been demonstrated by colony formation and cell proliferation assay (Moh et al., 2005a, b, 2008). A study on cell cycle profile showed that hepaCAM arrests cell cycle at the G2/M phase (Moh et al., 2008). Interestingly, hepaCAM induces cellular senescence as defined by the enlarged cell morphology and increased β-galactosidase activity (Moh et al., 2008). The underlying mechanism is unclear but clues can be obtained from the experimental data that hepaCAM elevates the expression levels of p53, p21 and p27, which are senescence-associated. Furthermore, knock-down of p53 results in reduction of p21 and alleviates senescence, suggesting that hepaCAM-induced cellular senescence is through the p53/p21 pathway (Moh et al., 2008). The suppressive effect of hepaCAM on cell growth together with the frequent loss of expression in human cancers fulfills two of the most important criteria to define tumor suppressors.
1.5.7.2 The effects of hepaCAM on cell-ECM interaction

Besides cell growth control, as a cell adhesion molecule, hepaCAM plays important roles in cell adhesion. Although hepaCAM does not clearly affect cell-cell adhesion (Moh et al., 2005b), it is capable of modulating cell-ECM adhesion. Cell spreading is significantly accelerated in hepaCAM-expressing HepG2 and MCF7 cells (Moh et al., 2005a, b), while cell detachment is delayed (Moh et al., 2005b). Boyden chamber and wound healing assays revealed that hepaCAM increased motility of HepG2 cells (Moh et al., 2005a). Similar studies were performed on MCF7 cells. Due to the poor invasive nature of MCF7 cells, barely any cell migrated through the transwell membrane. However wound healing assay showed significant increase of cell motility by hepaCAM (Moh et al., 2005b).

1.5.7.3 Interaction between hepaCAM and actin

hepaCAM has been shown to be partially insoluble in Triton X-100 (Moh et al., 2009b), indicating an association with the cytoskeleton. Immunofluorescence revealed the co-localization of hepaCAM and actin at cell boundaries, and co-immunoprecipitation confirmed a direct interaction between hepaCAM and actin (Moh et al., 2008). Intriguingly, an intact hepaCAM is needed to establish a stable interaction with F-actin. Deletion of any of its domains (1st Ig, 2nd Ig, and cytoplasmic) would result in complete loss of the interaction. In addition, an intact hepaCAM is also required to modulate cell-ECM adhesion and cell motility as deletion of the 1st Ig, 2nd Ig or cytoplasmic domain clearly diminishes the effects of hepaCAM (Moh et al., 2008).
1.5.7.4 Interaction between hepaCAM and caveolin-1

Besides F-actin, hepaCAM has been reported to be associated with caveolin-1, an essential structural component of caveolae, through the 1st Ig domain (Moh et al., 2009a). Since lipid rafts and its derivative caveolae serve as platforms for the assembly of protein complexes involved in cell signaling, the localization of hepaCAM in caveolae suggests a role of hepaCAM in signaling. Interestingly, caveolin-1 has a regulatory role on hepaCAM that re-expression of caveolin-1 promotes the expression and caveolar-recruitment of hepaCAM. Although the functional significance of this interaction remains to be determined, it suggests that caveolin-1 may play a role in the cross-talk of hepaCAM with cell signaling processes (Moh et al., 2009a).

1.5.8 hepaCAM in the central nervous system

One of our recent studies showed that hepaCAM is able to induce differentiation of the human glioblastoma U373-MG cells (Lee et al., 2009). hepaCAM significantly increases the expression of the astrocyte differentiation marker glial fibrillary acid protein and induces morphological changes characteristic of glioblastoma cell differentiation (i.e. small cell bodies with long, thin processes). Similar to the effects on HepG2 and MCF7 cells, hepaCAM inhibits cell proliferation and accelerates cell-ECM adhesion of U373-MG. However, cell motility is decreased by hepaCAM as demonstrated by wound closure assay and Boyden chamber assay (Lee et al., 2009), suggesting that hepaCAM has different functional roles in different cell types, probably due to distinct post-translational modification and/or protein-protein interactions.
The novel immunoglobulin-like cell adhesion molecule hepaCAM has been recently discovered and characterized. It is widely expressed in normal human tissues and often down-regulated in a variety of tumors. Restoration of hepaCAM in cancer cell lines leads to inhibition of cell growth and promotion of cell-ECM interaction. The frequent loss of hepaCAM in human cancers and the inhibitory effect of hepaCAM on cell growth suggest its role as a putative tumor suppressor.

The present study aims to further investigate the characteristics of hepaCAM. Two main subjects will be embraced: firstly, the molecular basis that underlies the hepaCAM-mediated cell-ECM, with an emphasis on the integrin-mediated signaling; and secondly, the proteolytic processing of hepaCAM.

The hypotheses are:

1. hepaCAM mediates cell-ECM interaction through affecting the integrin-mediated signaling
2. hepaCAM undergoes a proteolytic cleavage that is functionally important

The principal objectives of this study include:

1. To explore the interaction between hepaCAM and integrins;
2. To elucidate the effects of hepaCAM on the down-stream signaling events of integrins as well as cellular behaviors;
3. To delineate the enzymatic mechanisms that underlie hepaCAM cleavage;
4. To investigate the functional significance of hepaCAM cleavage.
CHAPTER 3  MATERIALS AND METHODS

3.1  Cell cultures

3.1.1  Cell line and culture condition

The human breast carcinoma MCF7 cells were purchased from the American Type Culture Collection (Manassas, VA) and maintained in an incubator at 37°C with 5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Life Technologies, Gaithersburg, MD).

3.1.2  Passaging cells

Cells were subcultured when they reached 80-100% confluence. The culture medium was removed and the cells were washed once with 1× phosphate buffered saline (PBS). To cells in T75 flasks, 2 ml of 1× trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco BRL) was added and incubated at 37°C for 2-5 min or until the cells were detached. Approximately 5 ml of culture medium was added to the flask and the cells in clumps were dispersed by repeated pipetting. Five ml of cells were removed and fresh medium was added to the remaining cells in the flask to a final volume of 10 ml. If cells were to be counted, 50 µl of the cell suspension was mixed with 50 µl of 0.8 mM trypan blue in PBS on a piece of parafilm. The mixture was then applied to a haemocytometer and at least 100 cells were counted. The number of cells/ml was calculated using the following formula:
Number of cells/ml = the average count/square of haemocytometer × the dilution factor × 10^4

3.1.3 Storing cells

Trypsinized cells were pelleted at 1,000 rpm for 5 min. The supernatant was discarded and the cells were resuspended in fresh freezing medium made up of 8% dimethyl sulfoxide (DMSO) in culture medium containing 10% FBS. The concentration of cells should be about 2 × 10^6 cells/ml of freezing medium. One ml-aliquot of the suspension was dispensed into each cryotube and stored at -80°C for overnight before transferring to liquid nitrogen for long-term storage.

3.2 Determination of nucleic acid concentration

The concentration and purity of DNA and RNA were determined by measuring their optical density (OD) at 260 and 280 nm. The concentration of DNA or RNA in a diluted solution was calculated using the molar extinction coefficient (OD_{260} = 1 = 50 µg/µl for DNA or OD_{260} = 1 = 40 µg/µl for RNA) as given below.

\[
\text{OD}_{260} \times \text{dilution factor} \times 50 \ \mu\text{g/µl} = \text{Concentration of DNA} \ \mu\text{g/µl}
\]

\[
\text{OD}_{260} \times \text{dilution factor} \times 40 \ \mu\text{g/µl} = \text{Concentration of RNA} \ \mu\text{g/µl}
\]

The ratio \(\text{OD}_{260}/\text{OD}_{280}\) was determined to estimate the purity of nucleic acids. Pure DNA or RNA has a ratio of 1.8-2.0. A ratio less than 1.8 indicates that there may be
proteins and/or other UV (ultraviolet) absorbers the sample. A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol.

3.3 Isolation of total RNA from cells

Total RNA was prepared from cultured cells using the RNeasy Kit (Qiagen, Hilden, Germany), and when necessary, contaminating DNA was digested on-column using the RNase-Free DNase Set (Qiagen). Cells were resuspended in 600 µl of β-mercaptoethanol-containing RLT buffer and homogenized by repeated pipetting or by passing through a 20-guage needle fitted to syringe. One volume of 70% ethanol was then added to the lysate and mixed. The mixture was applied to an RNeasy spin column with a 2-ml collection tube and centrifuged. The flow-through was discarded and 350 µl of buffer RW1 was pipetted into the spin column. After centrifugation, the spin column was transferred to a new 2-ml collection tube and washed twice with 500 µl of buffer RPE. The RNA was then eluted into a fresh collection tube from the spin column by adding 30 µl of RNase free water and centrifuging at 10,000 rpm for 1 min. Unless otherwise stated, all centrifugations were carried out at 10,000 rpm for 15 sec.

Digestion of DNA could be carried out during RNA extraction. After immobilization of RNA on the spin column and washing with buffer RW1, DNase I mix containing 10 µl (30 units) of DNase I stock solution and 70 µl of buffer RDD was added directly onto the spin column membrane and incubated at room temperature for 20 min. The reaction was terminated by the addition of 350 µl of buffer RW1 for 5 min. After centrifugation, the column was washed with buffer RPE as described above.
To ensure the integrity of RNA, samples of the purified RNA were separated by gel electrophoresis using standard 1% agarose gels containing ethidium bromide as described in Section 3.7. The 28S and 18S ribosomal RNAs should appear as distinct bands on the stained gel. In addition, the intensity of the 28S ribosomal RNA band should be approximately twice the amount of the 18S RNA.

### 3.4 Semi-quantitative RT-PCR

RT-PCR was performed to examine the mRNA expression of specific genes or to isolate the sequence of a gene for molecular cloning. RT-PCR was performed using the OneStep RT-PCR kit (Qiagen).

**TABLE 3-1 Primers for RT-PCR**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>hepaCAM:</strong></td>
<td></td>
</tr>
<tr>
<td>hCAM-F</td>
<td>5’ccatcaccgacgacacctc 3’</td>
</tr>
<tr>
<td>hCAM-R</td>
<td>5’gggtttctcgcctgce 3’</td>
</tr>
<tr>
<td><strong>I-CAM:</strong></td>
<td></td>
</tr>
<tr>
<td>I-CAM-F</td>
<td>5’ccggaaggtgtatgaactg3’</td>
</tr>
<tr>
<td>I-CAM-R</td>
<td>5’tccatggtgatctctc3’</td>
</tr>
<tr>
<td><strong>Integrin β1:</strong></td>
<td></td>
</tr>
<tr>
<td>Int β1-F</td>
<td>5’ aaggatccaccatgaattttacaccatgctc3’</td>
</tr>
<tr>
<td>Int β1-R</td>
<td>5’ tctagatccactttccctactcttctgc3’</td>
</tr>
<tr>
<td><strong>Housekeeping gene:</strong></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH:</strong></td>
<td></td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>5’cggatgtgggttggttgggc3’</td>
</tr>
</tbody>
</table>
GAPDH-R 5’ggcagagatgatgaccttttg3’

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>11</td>
</tr>
<tr>
<td>5× One-step RT-PCR buffer</td>
<td>5</td>
</tr>
<tr>
<td>dNTP Mix (10 mM)</td>
<td>1</td>
</tr>
<tr>
<td>Primer 1 (10 µM)</td>
<td>1.5</td>
</tr>
<tr>
<td>Primer 2 (10 µM)</td>
<td>1.5</td>
</tr>
<tr>
<td>DNase-treated RNA (50 ng/µl)</td>
<td>4</td>
</tr>
<tr>
<td>One-step RT-PCR Enzyme Mix</td>
<td>1</td>
</tr>
<tr>
<td>Final volume</td>
<td>25</td>
</tr>
</tbody>
</table>

Q solution was added into the reaction for amplifying template with high GC content.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT</td>
<td>50 or 55°C</td>
<td>30 min</td>
<td>1</td>
</tr>
<tr>
<td>(55°C for GC-rich template)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inactivation of RT</td>
<td>95°C</td>
<td>15 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
<td>25-35</td>
</tr>
<tr>
<td>Annealing</td>
<td>50-60°C</td>
<td>30 sec</td>
<td></td>
</tr>
</tbody>
</table>
### 3.5 Preparation of CaCl₂ competent *E. coli* cells

*E. coli* strain JM109 frozen stock was streaked onto a Luria broth (LB) agar plate and incubated overnight at 37°C. Following the protocol obtained from the Current Protocols in Molecular Biology, a single bacterial colony was inoculated into 50 ml of LB medium and cultured overnight at 37°C with shaking at 250 rpm. Two ml of bacterial culture was added into 200 ml of pre-warmed LB medium and grown to an OD₅₉₀ of 0.375. The bacteria were aliquoted into four 50-ml pre-chilled, sterile polypropylene tubes and incubated on ice for 10 min. The cells were harvested by centrifugation without brake at 3,000 rpm for 7 min at 4°C. Each pellet was gently resuspended on ice in 10 ml of ice-cold CaCl₂ solution followed by centrifugation at 2,500 rpm for 5 min at 4°C. A second resuspension in 10 ml of ice-cold CaCl₂ solution was carried out. The cells were incubated for 30 min on ice before centrifugation. Finally, each pellet was resuspended in 2 ml of ice-cold CaCl₂ solution and dispensed in 100 µl- aliquots into pre-chilled sterile 1.5-ml microcentrifuge tubes before storing at -80°C.

### 3.6 Restriction endonuclease digestion of DNA

Most restriction digestions were generally carried out in a final volume of 20 µl or 50 µl containing the DNA, restriction enzymes, bovine serum albumin (BSA) and applicable buffer as supplied by Promega. The reactions were incubated for a
minimum of 1 h at 37°C. Double digests were carried out simultaneously unless the buffer conditions were not suitable for both enzymes. In the latter cases, one digest was performed, purified with the QIAquick PCR Purification kit (Qiagen), and followed by the second enzyme digest.

3.7 **Agarose gel electrophoresis**

DNA or RNA was usually separated on a 1% agarose gel. To prepare a 1% SeaKem LE agarose (BioWhittaker, Walkersville, MD) gel, 0.5 g of agarose was added to 50 ml of 1× TAE buffer and microwaved until the agarose was completely dissolved. The agarose mixture was cooled under running tap water to about 45°C before adding 2.5 µl of 10 mg/ml ethidium bromide (Biorad, Richmond, CA). The mixture was then poured into a cast with a well comb and allowed to solidify. Once set, the comb was removed and the gel was submerged in 1× TAE buffer in the electrophoresis tank. Samples were mixed with gel loading buffer prior to application to the wells. The gel was then electrophoresed at 120 V for approximately 45 min. The nucleic acid was visualized under UV light.

3.8 **Purification of DNA from agarose gel**

The desired DNA fragment was quickly excised under UV light and the DNA was recovered by centrifuging the gel slice over siliconized glasswool in a 0.6-ml microcentrifuge tube placed in a 1.5-ml microcentrifuge tube. The DNA eluant was purified using either the QIAquick PCR Purification kit (Qiagen). Briefly, 5 volumes of buffer PB were added to 1 volume of the eluant. The mixture was transferred into a
QIAquick spin column and centrifuged. The column was washed with 750 µl of buffer PE. Subsequently, DNA was eluted with 10-50 µl of H₂O. All centrifugation steps were carried out at 13,000 rpm for 1 min at room temperature.

3.9 DNA ligation

In a ligation reaction, T4 DNA ligase (Promega) covalently links the phosphodiester bonds between the insert DNA and the vector DNA to join the two fragments together. Ligation was performed in a total volume of 10 µl containing appropriate molar ratios of vector and insert DNA (usually 1:2), 1 µl T4 DNA ligase and 1× ligase buffer, and incubated either for 1-2 h at room temperature or overnight at 4°C.

3.10 Transformation of E. coli

Transformation facilitates the uptake and expression of DNA by a living cell. Ten µl of ligation mix was added to 100 µl of JM109 competent cells, mixed, and left on ice for 30 min. The cells were then heat-shocked at 42°C for 45 sec before rapidly returning the tube to ice for 2 min. After addition of 900 µl of LB medium, the bacteria were incubated with shaking at 150 rpm for 90 min at 37°C. The cells were then pelleted by centrifugation at 2,000 rpm for 3 min. Excess supernatant was discarded, leaving approximately 200 µl in the tube. The cells were then resuspended and plated onto LB agar plates containing the appropriate antibiotic. For blue/white selection, the LB agar plates were spread with 20 µl of X-Gal and 100 µl of IPTG prior to plating of bacteria. The plates were incubated for 18-24 hours at 37°C.
3.11 **Plasmid miniprep**

A bacterial colony or an aliquot of bacterial glycerol stock was inoculated into 3 ml of LB medium supplemented with the appropriate antibiotic. The inoculated bacteria were cultured at 37°C overnight with shaking at 250 rpm. Two ml of bacteria were pelleted by centrifugation at 13,000 rpm for 1 min. Plasmid extraction was carried out using the QIAprep® Miniprep kit from Qiagen according to the manufacturer’s instruction. The pellet was resuspended in 250 µl of buffer P1 before lysing in 250 µl of buffer P2. The mixture was gently inverted 4-6 times till a clear and viscous lysate was obtained. Unwanted materials including bacterial cell wall fragments, proteins, and chromosomal DNA were precipitated by the addition of 350 µl of buffer N3 and centrifugation at 13,000 rpm for 10 min. The clear supernatant containing plasmid DNA was transferred to a spin column and centrifuged at 13,000 rpm for 1 min. The column was washed with 500 µl of buffer PB and 750 µl of buffer PE. Plasmid DNA was then eluted with 50 µl of sterile H2O and stored at -20°C.

3.12 **Bacterial glycerol stock**

Glycerol was diluted to 50% in H2O and filter-sterilized. Three hundred and fifty µl of bacterial culture was mixed with 150 µl of 50% glycerol and stored at -80°C.

3.13 **Automated DNA sequencing**

DNA Sequencing reactions were performed with the ABI PRISM Big Dye Reaction Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) according to the manufacturer’s instruction.
Table 3-4  Primers for sequencing

<table>
<thead>
<tr>
<th>Primer name</th>
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</tr>
</thead>
<tbody>
<tr>
<td><em>hepaCAM</em>-specific:</td>
<td></td>
</tr>
<tr>
<td>hCAMMint-F</td>
<td>5’caatgactcagaatgcctgtcc 3’</td>
</tr>
<tr>
<td>hCAMMint-R</td>
<td>5’cttccgttcctgcctcacttc3’</td>
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</table>

Table 3-5  Sequencing components

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double-stranded plasmid</td>
<td>300-500 ng</td>
</tr>
<tr>
<td>Big Dye terminator mix</td>
<td>4</td>
</tr>
<tr>
<td>5× Sequencing buffer</td>
<td>2</td>
</tr>
<tr>
<td>Primer (10 µM)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Final volume Add H₂O to a final volume of 20 µl

Table 3-6  Sequencing conditions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>96°C</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>5 sec</td>
<td>25</td>
</tr>
<tr>
<td>Extension</td>
<td>60°C</td>
<td>4 min</td>
<td></td>
</tr>
</tbody>
</table>

The sequencing product was precipitated with 2 µl of 125 mM EDTA, 2 µl of 3 M sodium acetate and 50 µl of absolute ethanol for 15 min at room temperature before centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was washed in 250 µl of 70% ethanol and centrifuged at 13,000 rpm for 5 min at 4°C. The final pellet was air
dried before being reconstituted in sequencing loading buffer and analyzed on an ABI PRISM™ TM 337 automated sequencer (Applied Biosystems).

### 3.14 Transient and stable transfection

Cells used for transfection were seeded one day before transfection at a density such that they would be 40-70% confluent on the day of transfection. Transfections were carried out with the reagent of Lipofectamine Plus (Invitrogen) according to the manufacturer’s instructions. Cells were transfected in serum-free DMEM with DNA precomplexed with PLUS and Lipofectamine reagents for 3-5 h, after which, the medium containing the complexes was removed and replaced with DMEM medium supplemented with 10% FBS.

For stable transfections, cells transfected in 35-mm plates were reseeded into 100-mm plates or T75 flasks after 24 h of transfection and selected under 800 µg/ml of G418 (Sigma) or 10 µg/ml of blasticidin (Invitrogen) for 2 weeks. Thereafter, the cells were trypsinized and each cell picked under microscope was transferred into a well of a 24-well plate containing parental cells at 20% confluence. Parental cells were added to stimulate the growth of the stable cell. The cells were then allowed to propagate in culture medium without antibiotics for 2 days before replacing with selection medium. The cells were kept in selection medium until all the parental cells were killed and the stable clone reached confluence. Alternatively, after trypsinizing the stable cells, the cells were seeded at very low density to allow it to grow into a small colony of about 40 cells. Under microscope, the colony was gently detached and aspirated using a pipette tip and transferred to a 24-well plate. The cells were maintained until
confluent in selection medium. Once the cells were confluent, a fraction of them was used for Western blot analysis and immunocytochemistry to confirm the expression of the desired protein and cell homogeneity, respectively. The positive clones were subsequently expanded.

For transient transfections, the transfected cells were cultured without antibiotic selection for 24-48 hours before assaying.

3.15 Immunocytochemistry

3.15.1 Indirect immunofluorescence

Cells grown on coverslips were washed twice with PBS and fixed with 3.7% paraformaldehyde for 15 min at 37°C. The subsequent steps were all carried out at room temperature. Following three 5-min washes with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS for 5 min. A second set of washing was carried out and the nonspecific sites on coverslips were blocked in 1% BSA in PBS. The cells were then incubated for 1 h with primary antibody diluted in 1% BSA, washed, and incubated for 1 h with Alexafluor-conjugated secondary antibody (Molecular Probes, Eugene, OR) diluted in 1% BSA. Excess secondary antibody was removed by washing 3 times in PBS. After a final rinse in H2O, the coverslips were mounted onto the microscopic slides with FluorSave reagent (Calbiochem, La Jolla, CA), and analyzed by fluorescence microscopy (Carl Zeiss, Germany) or confocal microscopy (FV1000, Olympus, Japan).
3.15.2 Labeling of F-actin

F-actin is efficiently labeled with fluorescent phalloidin conjugates. Phalloidin is a fungal toxin produced by the poisonous mushroom *Amanita phalloides* that acts by binding to and stabilizing F-actin. Stock solution of tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin (Sigma) was prepared in DMSO at 1 mg/ml. F-actin of fixed and permeabilized cells was labeled with 1 µg/ml of TRITC-conjugated phalloidin diluted in PBS for 1 h at room temperature.

3.16 MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay measures cell viability. The assay is based on the reduction of the yellow MTT into purple formazan crystals catalyzed by enzymes produced only in metabolically active live cells.

To evaluate cell viability, a volume of 100 µl of MTT (Roche; 5 mg/ml in PBS) was added to cells transfected for 48 h in a 6-well culture plate. After 3 h of incubation at 37°C, cells were lysed and formazan crystals were dissolved in 1 ml of isopropanol with 0.4 N HCl. Wells containing all admixtures except cells were used as blanks. OD was read at 570 nm using an ELISA reader. Cell viability (CV) was computed by the following equation:

\[
CV(\%) = (1 - \frac{OD\text{-}c - OD\text{-}e}{OD\text{-}c}) \times 100\%
\]
where OD_{e} = OD reading of experiment; OD_{c} = OD reading of control. Cell viability of control was regarded as 100%.

To assess the rate of cell proliferation, $2 \times 10^4$ cells were seeded and cultured in six 6-well plates in triplicates. MTT assay was carried out once the cells had attached to establish the base line of cell growth. At every 24 h of cell culture for 5 days, MTT assay was performed to evaluate the cell viability. The growth rate of each cell line was presented as fold of increase in cell viability against the respective base line obtained on the day of seeding cells.

3.17 Colony formation

This assay was performed to evaluate the effect of the gene of interest on cell growth in colony form. Transfected cells were cultured in selection medium containing 10 µg/ml of blasticidin or 800 µg/ml of G418 for 2 weeks without trypsinization while medium was refreshed every 2 days or 3-4 days, respectively. The cell colonies formed at the end of experiment were visible and the number of colonies was counted.

3.18 Cell cycle analysis

Cells were fixed in 70% ethanol and stored overnight at -20°C. Immediately before analysis, the cells were re-suspended in staining solution (200 µg/ml propidium iodide, 0.1% Triton X-100 and 2 mg/ml RNase A) and incubated for 30 min at room temperature. Cell cycle distribution was determined using a Beckman Coulter Epics
Altra flow cytometer (Germany). Data were analyzed using the WinMDI (Joseph Trotter) software version 2.8.

### 3.19 Cell adhesion assay

For cell attachment assay, plates were coated with fibronectin (10 µg/ml; Santa Cruz) and allowed to polymerize for 1 h at 37°C or at room temperature. The plates were then blocked by 1% BSA for 1 h. Cells were seeded and allowed to adhere. After 5 min, unattached cells were washed away by 1× PBS. Attached cells were stained by 1% crystal violet. After air-dried, the stained cells were desolved in 1% SDS and optical absorbance of the solution at wavelength 490 nm was measured.

To examine cell spreading, cells were seeded onto the coated coverslips and incubated under standard culture conditions. Cell morphology was observed by microscopy. Unspread cells were defined as round cells while spread cells were defined as cells with extended processes. The percentage of cells demonstrating spread morphology was quantified in 10 randomly selected fields.

### 3.20 Wound healing assay

Cell migration was assessed by the wound healing experiment on monolayer cells. Cells were seeded in 35-mm culture plates at high density and allowed to form confluent monolayers overnight. Wounds were made by sterile plastic 200-µl micropipette tips and allowed to be healed in culture medium containing 10% FBS. Microscopic pictures representing the changes in diameter ($D$) of each wound were
taken at 24 and 48 hours. By measuring the remaining gap space on the pictures, the percentage of wound closure was computed into ratio \( \frac{D_{24/48h}}{D_{\text{initial}}} \times 100\% \).

### 3.21 Chemical crosslinking

A monolayer or a single suspension of cells were incubated in PBS containing 2 mM dithiobis(succinimidyloxydipropionate) (DSP; Pierce) dissolved in DMSO or 3,3′-Dithiobis (sulphosuccinimidyl propionate) (DTSSP; Pierce) dissolved in H₂O at room temperature for 30 min. The reaction was quenched with the addition of 20 mM Tris-HCl pH 7.5 for 15 min. Single-cell suspension was assured by microscopic observation before and after chemical crosslinking reaction. DTSSP-crosslinked proteins were resuspended in Laemmli sample buffer without reducing agent 50 mM dithiothreitol (DTT) unless indicated. Cell lysate was prepared in radioimmunoprecipitation assay (RIPA) buffer containing 10 mM iodoacetamide to inhibit formation of nonspecific disulfide linkages.

### 3.22 Preparation of whole cell extracts

Cells were scraped in RIPA buffer containing protease inhibitors followed by incubation on ice for 15 min. The cell lysate was cleared by centrifugation at 13,000 rpm for 10 min at 4°C. Protein samples were mixed with an equal volume of 2 × or \( \frac{1}{4} \) volume of 5 × Laemmlni sample buffer and boiled for 5 min before Western blot analysis.
3.23 Subcellular fractionation

Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit (Thermo Scientific, Waltham, MA) according to the manufacturer’s instruction. Briefly, cells were harvested and the cell pellet was incubated with Cytoplasmic Extraction Buffer at 4°C for 10 min. After centrifugation at 500× g for 5 min, the supernatant containing the cytoplasmic proteins was stored and the remaining pellet was incubated with Membrane Extraction Buffer at 4°C for 10 min. The supernatant after centrifugation at 3,000× g for 5 min was the membrane extract. The remaining pellet was incubated with Nuclear Extraction buffer at 4°C for 30 min with vortex to extract soluble nuclear proteins after centrifugation at 5,000× g for 5 min. The chromatin-bound proteins were extracted by incubating the remaining pellet with Nuclear Extraction Buffer plus CaCl₂ and Micrococcal Nuclease at room temperature for 15 min followed by vortex and centrifugation at 16,000× g for 5 min. The remaining pellet was incubated with Pellet Extraction Buffer containing protease inhibitors at room temperature for 10 min followed by vortex. After centrifugation at 16,000× g for 5 min, the supernatant was the cytoskeletal extract.

3.24 in vitro translation

Pure proteins of hepaCAM and integrin β1 were obtained by in vitro translation using TNT® Quick Coupled Transcription/Translation Systems (Promega) and Human In Vitro Protein Expression Kit (Pierce, Rockford, IL), respectively, according to the instructions of the manufacturers. Briefly, when using the Promega kit, components listed in Table 3-7 were mixed gently to prepare a reaction. The reaction was incubated at 30°C for 60-90 min followed by Western blot analysis for protein
expression or immunoprecipitation. For the Pierce kit, components listed in Table 3-8 were mixed gently to prepare a 20 µl transcription reaction. The reaction was incubated at 32°C for 60-75 min. For translation reaction, components in Table 3-9 were assembled and incubated at 28-32°C for 90 min. The final product was subjected to analysis by Western blot or immunoprecipitation.

Table 3-7 Components for TNT® Quick Coupled Transcription/Translation Systems (Promega)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT® Quick Master Mix</td>
<td>40</td>
</tr>
<tr>
<td>Methionine, 1 mM</td>
<td>1</td>
</tr>
<tr>
<td>Plasmid DNA template</td>
<td>2</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>1-2</td>
</tr>
</tbody>
</table>

Final volume 20

Table 3-8 Components for transcription reaction (Human In Vitro Protein Expression Kit, Pierce)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
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<tbody>
<tr>
<td>Nuclease-free Water</td>
<td>8</td>
</tr>
<tr>
<td>5× Transcription Buffer</td>
<td>4</td>
</tr>
<tr>
<td>NTP Mix</td>
<td>4</td>
</tr>
<tr>
<td>Plasmid DNA template (0.5 µg/µl)</td>
<td>2</td>
</tr>
<tr>
<td>T7 RNA Polymerase</td>
<td>2</td>
</tr>
</tbody>
</table>

Final volume 20
Table 3-9 Components for translation reaction (Human *In Vitro* Protein Expression Kit, Pierce)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate for protein Expression</td>
<td>12.5</td>
</tr>
<tr>
<td>Accessory Proteins</td>
<td>2.5</td>
</tr>
<tr>
<td>Nuclease-free Water</td>
<td>3.75</td>
</tr>
<tr>
<td>Salt Solution A</td>
<td>1</td>
</tr>
<tr>
<td>Amino Acid minus Met</td>
<td>0.5</td>
</tr>
<tr>
<td>Amino Acid minus Leu</td>
<td>0.5</td>
</tr>
<tr>
<td>RNAse Inhibitor (optional)</td>
<td>1</td>
</tr>
<tr>
<td>Energy Mix</td>
<td>1.25</td>
</tr>
<tr>
<td>Target Protein Transcription Mix</td>
<td>2</td>
</tr>
<tr>
<td>Final volume</td>
<td>25</td>
</tr>
</tbody>
</table>

3.25 Determination of protein concentration by the Bradford method

The Bradford assay is a dye-binding assay based on the differential color change of Coomassie blue G dye as it binds to protein. In this study, the concentrations of proteins were determined by Bradford assay (Biorad) at OD$_{595}$.

One part of the Dye Reagent Concentrate was diluted in 4 parts of H$_2$O to constitute the working solution. Twenty µl of protein diluted in PBS was mixed with 1 ml of working dye solution and the absorbance measured. A standard curve was plotted by measuring the absorbance of BSA at concentrations ranging from 0.1 mg/ml (Figure 3-1). The concentrations of test proteins were then determined from this curve.
3.26 Western blot analysis

3.26.1 Separation of proteins by polyacrylamide gel electrophoresis

Denatured protein samples were loaded onto 10% sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gel and resolved by running in 1× SDS-PAGE running buffer at 150 V for 70 min or until the desired distance was reached.

3.26.2 Protein transfer

After electrophoresis, proteins were transferred from the gel onto polyvinylidene fluoride (PVDF) membrane (Biorad) using BioRad’s Mini Trans-Blot Electrophoretic Transfer Cell. The gel, membrane, Whatman filter papers and fiber pads were equilibrated in Towbin buffer for 15 min before assembly as illustrated in Figure 3-2.
The proteins were transferred from the gel (negative electrode) to the membrane (positive electrode) at 100 V for 60 minutes in Towbin buffer containing 0.1% SDS.

Figure 3-2 Assembly for protein transfer. (Source: instruction manual of Biorad’s Mini Trans-Blot Electrophoretic Transfer Cell)

3.26.3 Western blotting

The membrane was rinsed with 1× tris buffered saline (TBS) and blocked in blocking buffer (5% skim milk) for 1 h. Incubation with primary antibodies diluted in blocking buffer was carried out either at room temperature for 1 h or 4°C overnight. The primary antibodies used included mouse anti-V5 from Invitrogen, mouse anti-integrin β1 (clone P5D2, for immunoprecipitation) from Abcam (Cambridge, MA), rat anti-active integrin β1(clone 9EG7) from BD Biosciences (Sprks, MD), mouse anti-calpain 1 from Biomed Diagnostics (White City, OR), mouse anti-E-cadherin from Zymed Laboratories (San Francisco, CA), rabbit anti-integrin β1(for Western blot analysis), rabbit anti-integrin αV, rabbit anti-p-paxillin (Y118), rabbit anti-p-FAK (Y397), rabbit anti-talin from Millipore (Billerica, MA), mouse anti-integrin β1 (clone JB1B, for immunofluorescence), goat anti-integrin α2, goat anti-integrin α3,
rabbit anti-paxillin, rabbit anti-FAK, mouse anti-p53 and mouse anti-GAPDH from Santa Cruz. Blots were then washed three times in TBS/Tween 20 (TBS/T), 5 min each, and then incubated for 1 h in the appropriate horseradish peroxidase-conjugated secondary antibody (Santa Cruz). After four 5-min washes, the enzymatic activity was detected with chemiluminescence luminol reagent (Santa Cruz) or enhanced chemiluminescence (Thermo Scientific) and autoradiography. The band intensities were quantified with a GS-800 densitometer and QuantityOne software (Biorad).

3.26.4 Stripping of Western blot

The Western blot to be re-probed was stripped in two 15-min washes of stripping buffer. The membrane was then rinsed with 1× TBS before re-blocking.

3.27 Immunoprecipitation

Immunoprecipitation was used to purify the desired protein and co-immunoprecipitation was performed to detect protein-protein interactions. Cell lysate prepared in NP-40 buffer was precleared with Protein G-agarose beads (Santa Cruz) for 1 h at 4°C with agitation. The supernatant was recovered by centrifugation at 2,500 rpm for 1 min. The precleared lysate was then mixed with protein G-agarose beads and specific antibody, and incubated overnight at 4°C before washing four times with RIPA buffer or PBS when a reduced stringency was desired. The beads were resuspended in 2× Laemmli sample buffer, boiled for 10 min and the eluted proteins were subjected to Western blot analysis.
3.28  GTPase activity assay

Rho, Rac1, and Cdc42 activities were analyzed using the Rho and Rac1/Cdc42 activation assay kits (Cell Biolabs, San Diego, CA), respectively. Briefly, serum-starved cells were seeded onto fibronectin-coated Petri dishes and incubated for 30 min or 3 h at 37°C. Cell lysate was prepared according to the manufacturer’s instructions. Equal amount of protein per sample was incubated for 1 h at 4 °C with GST-sepharose loaded with either 400 µg of GST-Rhotekin-RBD or 20 µg of GST-PAK1-PBD. Bound proteins were separated by SDS-PAGE and analyzed by Western blot using the supplied monoclonal antibodies against Rho, Rac1 and Cdc42.

3.29  Time-lapse microscopy

Serum-starved cells were plated on fibronectin-coated 35-mm Petri dishes in DMEM and allowed to adhere and migrate. Cell migration was monitored using the time lapse imaging system BioStation IM-Q (Nikon, Japan) at 5-min intervals for 8 h. The positions of nuclei were tracked using NIS-Elements Ar (Nikon) to quantify cell motility and to calculate velocities and directionalities. Five cells were quantified in each view for 5 views for each cell type. Cell velocity was calculated as the total path length (l) divided by time duration (t); directionality was defined as the ratio of distance between start and end points (d) over the total path length (l).

3.30  Statistical analysis

All statistical analyses were performed with the software InStat 3.0 (GraphPad, San Diego, CA). One sample t test was used to compare the mean of one sample to the
normalized 1. Unpaired *t* test was used to compare the means of two groups. Nonparametric analysis of variance (ANOVA) was performed to compare the differences among more than two means. *P* < 0.05 was considered significant.
4.1 Connection between hepaCAM and the integrin-dependent signaling

4.1.1 Protein expression of hepaCAM in non-tumorous and tumorous breast cell lines

As introduced previously, hepaCAM is frequently down-regulated in cancer cells. To examine the regulation of hepaCAM in breast cancer cells, protein expression of hepaCAM in two normal breast cell lines MCF10-A and MCF12-A and one tumorous cell line MCF7 was examined by Western blot using anti-hepaCAM antibody. No expression of hepaCAM was detected in all cell lines including the normal ones (Fig 4-1). Although MCF10-A and MCF12-A are non-tumorous, they have been immortalized and may have altered gene expression compared to normal breast tissues. Thus hepaCAM expression may have been lost in the two cell lines.

4.1.2 Stable transfection of hepaCAM into MCF7 cells

Human breast carcinoma MCF7 cells were stably transfected with either pcDNA6B/V5-His or vector containing the entire hepaCAM sequence. Protein expression of hepaCAM in the two cell lines was examined by Western blot. A 75 kD band was detected in hepaCAM transfected cells while no protein expression was observed in the vector transfected cells (Fig 4-2). hepaCAM-expressing MCF7 cells were designated hepaCAM(+) and vector transfected cells were designated hepaCAM(-).
Figure 4-1 Protein expression of hepaCAM in non-tumorous and tumorous breast cell lines. Protein expression of hepaCAM in MCF7, MCF10-A and MCF12-A cells (left panel) was examined by Western blot using anti-hepaCAM antibody. MCF7 cells were stably transfected with pcDNA6/V5-His vector [hepaCAM(-)] or vector containing the hepaCAM sequence [hepaCAM(+) (right panel). The expression of hepaCAM in stable clones of MCF7 cells was examined by Western blot.
4.1.3  hepaCAM alters the cell-ECM interaction of MCF7 cells on a fibronectin substrate

4.1.3.1 hepaCAM accelerates initial cell attachment

Initial cell attachment refers to the adherence between cells and the binding substrate immediately after cell-ECM contact. This process is usually completed within minutes. The effects of hepaCAM on initial cell attachment were examined. hepaCAM(-) and hepaCAM (+) cells were plated on Petri-dishes coated with the ECM protein fibronectin. After 5 min, unattached cells were washed away by PBS and the attached cells were stained by crystal violet. The attachment rates of cells were derived from the OD readings at wavelength 570 nm compared with a 100% attachment control. As shown in Figure 4-2, the expression of hepaCAM increased the initial cell attachment by approximately 3 fold as compared to the control cells.

4.1.3.2 hepaCAM increases cell spreading

After initial cell attachment is cell spreading which is characterized by membrane protrusions in morphology. To examine the effects of hepaCAM on cell spreading, hepaCAM(-) or hepaCAM(+) stable clones of MCF7 cells were plated onto a fibronectin-coated surface. After 30 min, most of the control cells remained rounded while a significantly higher percentage of hepaCAM-transfected cells displayed numerous protrusions, exhibiting spread morphology, consistent with our previous results (Moh et al., 2005a, b). The percentage of hepaCAM transfected cells displaying a spread morphology was almost double that of the control cells (Fig. 4-3
Figure 4-2 hepaCAM promotes initial cell attachment. (A) hepaCAM(-) and hepaCAM(+) MCF7 cells were serum-starved overnight and seeded onto Petri-dishes coated with 10 μg/ml fibronectin. After 5 min, unattached cells were washed away with PBS and attached cells were stained with 1% crystal violet. Microscopic pictures were taken. Magnification, 320 ×. As a control, the cells were allowed to spread for 3 h and the cell density of the corresponding cell line was considered as 100%. The attachment rates of cells were derived from comparing the OD readings at wavelength 570 nm against the 100% control. (B) Bar charts depict the attachment rates. Data represent means ± SD. *, P < 0.05.
Figure 4-3 hepaCAM increases cell spreading. (A) hepaCAM(-) and hepaCAM(+) cells were allowed to adhere on fibronectin for 30 min and microscopic pictures were taken. Magnification, 320 ×. The percentage of cells demonstrating spread morphology was quantified in 5 randomly selected fields. (B) Bar charts depict the percentages of spread cells. Data represent means ± SD. *, $P < 0.05$. 
4.1.3.3 hepaCAM promotes cell migration

Cell migration involves repeated adhesion at the cell front and detachment at the cell rear. Such cyclic processes drive the cells to move forward. To examine if hepaCAM also affects cell migration, motility of hepaCAM(-) and hepaCAM(+) cells on fibronectin was monitored for 8 h by time-lapse microscopy under standard cell culture condition. The snapshot photos showed the morphology of vector or hepaCAM-transfected cells at 0, 2, 4, and 8 h (Fig. 4-4). hepaCAM-transfected cells displayed more extensive protrusions and lamellipodia than vector transfected cells during migration. The paths of migration of 5 representative cells of each cell line were tracked over a period of 8 h. The migration tracks revealed that the control cells were not very motile and their migration was random with low persistence. In contrast, a noticeable percentage of hepaCAM-transfected cells displayed substantial migration, leaving behind apparently longer migration paths (Fig. 4-5). The average cell velocity was increased approximately 5-fold by hepaCAM. The directionality was also significantly increased, indicating a higher persistence of cell migration.

4.1.4 Interaction between hepaCAM and integrin β1

4.1.4.1 Integrin β1 is the major molecule mediating cell-ECM adhesion of MCF7 cells

Cell adhesion is strictly dependent on the interaction between the ECM substrate and the adhesion molecules expressed on the cell surface. For most cell types, integrins are the major receptors that mediate cell-ECM adhesion (Hynes, 2002). Integrin β1 is the most highly expressed integrin in MCF7 cells (Doerr and Jones, 1996) and also an essential component of the fibronectin receptor. Both vector- and hepaCAM-transfected cells were treated with either non-specific mouse IgG or an integrin β1
Figure 4-4 Time-lapse microscopy of migration of hepaCAM(-) and hepaCAM(+) cells. hepaCAM(-) and hepaCAM (+) cells were seeded on fibronectin-coated surface. The migration of cells was monitored for 8 h at 5-min intervals by a Nikon BioStation. Snapshots were taken at 0, 2, 4 and 8 h after seeding.
Figure 4-5 The migration tracks, velocity and directionality of hepaCAM(-) and hepaCAM(+) cells. (A) The migration paths of 5 cells/view for 5 views of each cell line were tracked over 8 h. (B) The cell velocity was calculated by dividing the total path length by the time of duration. Directionality was determined as the ratio of the linear distance from the end point to the origin (d) over the total path length (l). Data represent means ± SD. *, $P < 0.05$. 
function blocking antibody (clone P5D2) and spread on fibronectin. After 1.5 h, both IgG treated vector- and hepaCAM-transfected cells were almost fully attached, and hepaCAM transfectants were much more spread than the control cells. However, when integrin $\beta_1$ function was blocked, the attachment of both vector- and hepaCAM-transfectants was almost abolished (Fig. 4-6), indicating that integrin $\beta_1$ is indeed the major molecule mediating cell-ECM adhesion of MCF7 cells. It also suggested that hepaCAM alone is not able to mediate cell-ECM adhesion.

**4.1.4.2 hepaCAM and integrin $\beta_1$ are physically associated**

It has been reported that integrins are able to interact with other adhesion molecules such as the Ig superfamily of CAMs that hepaCAM belongs to (Brummer et al., 2001). To examine if hepaCAM interacts with integrin $\beta_1$, co-immunoprecipitation was performed. The results showed no interaction between hepaCAM and integrin $\beta_1$ was detected (Fig. 4-7, left panel). To strengthen the possible protein-protein interaction and avoid loss of signal during the stringent washing steps, the cells were treated with DSP, a cross-linking agent, before co-immunoprecipitation. Finally, hepaCAM was successfully detected in the integrin $\beta_1$ immunoprecipitate using this method (Fig. 4-7, right panel). The data indicated that hepaCAM and integrin $\beta_1$ coexist in one complex but the association may be relatively weak.

**4.1.4.3 cis/trans interaction between hepaCAM and integrin $\beta_1$**

We then tested whether the association was in *cis* or *trans*. *Cis* is the conformation when two molecules interact on the same cell, while *trans* refers to the interaction of two molecules of two different cells. hepaCAM-transfected cells were grown into a
Figure 4-6 Integrin β1 is the dominant integrin in MCF7. (A) The cells were treated with either 1 μg/ml mouse IgG or integrin β1 funcion blocking antibody and spread on fibronectin coated surfaces for 1.5 h. Magnification, 320×. (B) The floating cells were washed away by PBS and the attached cells were stained with 1% crystal violet and OD measured at 570 nm. Attachment of IgG treated cells was considered 100% for each cell line. Attachment of cells treated with integrin β1 funcion blocking antibody was calculated accordingly. Data represent means ± SD. ***, P < 0.001.
Figure 4-7 Co-immunoprecipitation of hepaCAM and integrin β1. Cell lysate prepared from hepaCAM(+) cells was treated without (left) or with DSP (right) and immunoprecipitated with either normal IgG or anti-integrin β1 antibody. The immunoprecipitates were then analyzed with anti-V5 (to detect hepaCAM) or anti-integrin β1 antibody.
monolayer or disaggregated into single-cell suspension. The cells were then treated with DTSSP, a cross-linking agent that is impermeable to cell membranes, to cross-link proteins on the cell surface before lysis. The results showed that hepaCAM was co-immunoprecipitated with integrin β1 from the cell lysate of cells in both monolayer and single-cell suspension (Fig. 4-8), indicating that hepaCAM and integrin β1 interact with each other on the same cell. Since this method was only able to confirm cis interaction, the possibility of trans interaction was not excluded.

### 4.1.4.4 Direct/indirect interaction between hepaCAM and integrin β1

Next, we studied whether the interaction was direct or indirect. Pure hepaCAM and integrin β1 proteins were obtained by in vitro translation. hepaCAM was translated using a rabbit reticulocyte translation system (Promega) while integrin β1 was translated from a human translation system (Pierce). The expression of the resulted proteins was examined by Western blot analysis (Fig. 4-9 A). While the translated integrin β1 was ~130 kD, almost identical to the endogenous integrin β1 expressed in MCF7 cells, the translated hepaCAM was ~50 kD, smaller than the transfected hepaCAM in MCF7 cells, probably due to different posttranslational modification. The obtained hepaCAM and integrin β1 proteins were mixed and incubated overnight at 4°C before immunoprecipitation. No association between in vitro translated hepaCAM and integrin β1 proteins was found (Fig. 4-9 B), suggesting that the interaction between hepaCAM and integrin β1 might be indirect.
Figure 4-8 *cis*-interaction between hepaCAM and integrin β1. A monolayer or a single cell suspension of hepaCAM transfectants was crosslinked with DTSSP, lysed and immunoprecipitated with anti-integrin β1. The immunoprecipitate was analyzed by Western blot.
Figure 4-9 Direct/indirect interaction between hepaCAM and integrin β1. (A) Pure integrin β1 and hepaCAM proteins were obtained by *in vitro* translation system and the protein expression was examined by Western blot using anti-integrin β1 and anti-V5 antibodies, respectively. (B) The interaction between in vitro translated hepaCAM and integrin β1 was examined by co-immunoprecipitation.
4.1.5 Domain dependence of hepaCAM-integrin β1 interaction

4.1.5.1 Generation of truncated mutants of hepaCAM

Several truncated mutants were generated to study the dependence of hepaCAM-integrin β1 interaction on different domains of hepaCAM. The amino acid sequences of the 1st Ig, the 2nd Ig, and the cytoplasmic domain of hepaCAM are denoted in Figure 4-10 A using distinct colors. The mutant hCAMΔ1st Ig was generated by deleting residues 33-145, hCAMΔ2nd Ig by deleting residues 155-227 and hCAMΔtail by deleting residues 33-227. The signal peptide was retained at the N-terminus of each mutant (Fig. 4-10 B). The sequences were cloned into the eukaryotic expression vector pcDNA6B/V5-His.

4.1.5.2 Protein expression of hepaCAM mutants

The protein expression of the truncated mutants of hepaCAM was examined by Western blot analysis (Fig. 4-11). Given the same amounts of total cell lysate, the expression level of hCAMΔ1st Ig was relatively low compared to wild-type (WT) hepaCAM and other mutants, while hCAMΔtail had a very high expression. Interestingly, both hCAMΔ2nd Ig and hCAMΔtail exhibited two distinct bands. Previously it has been shown that hepaCAM is glycosylated on its extracellular domain (Moh et al., 2005b). Perhaps the thicker bands at a higher molecular weight of hCAMΔ2nd Ig and hCAMΔtail represented differentially glycosylated protein products. These data suggested that deletion of any of the domains would affect the level of protein expression of hepaCAM. Moreover, both hCAMΔ2nd Ig and hCAMΔtail
Figure 4-10 Truncated mutants of hepaCAM. (A) Protein sequence of hepaCAM. Different domains are represented by distinct colors. Box: signal peptide; Red: 1st Ig domain; Blue: 2nd Ig domain; Green: transmembrane domain; Pink: cytoplasmic domain. (B) Schematic representation of hepaCAM (wild-type), hCAMΔ1st Ig (with deletion of residues 33-145), hCAMΔ2nd Ig (with deletion of residues 155-227), and hCAMΔtail (with deletion of 33-227). □, signal peptide.
Figure 4-11 Protein expression of hepaCAM mutants. Plasmids containing wild type hepaCAM, hCAMΔ1st Ig, hCAMΔ2ndIg or hCAMΔtail were transfected into MCF7 cells. After 48 h, the expressed protein was examined by Western blot using anti-V5 antibody.
possess the 1st Ig domain while hCAMΔ1st Ig does not. Thus the first Ig domain probably contains the major site where varied glycosylation occurs.

4.1.5.3 Subcellular localization of hepaCAM mutants

Plasmids containing hepaCAM, hCAMΔ1st Ig, hCAMΔ2nd Ig, or hCAMΔtail were stably transfected into MCF7 cells. The subcellular localization of the proteins was revealed by immunofluorescence by staining the cells with anti-V5 antibody (Fig. 4-12). WT hepaCAM had a clear distribution on the plasma membrane, especially on the cell boundary, consistent with previous observations (Moh et al., 2005a, b). There was also some distribution in the cytoplasm, particularly in the perinuclear area. Interestingly, hCAMΔ1st Ig exhibited localization distinct from WT hepaCAM. The distribution on plasma membrane was completely lost. Instead, the protein was extensively distributed in the cytoplasm, highly resembling the pattern of endoplasmic reticulum. In contrast, hCAMΔ2nd Ig retained the plasma membrane distribution, showing a pattern similar to WT hepaCAM. However, the intracellular distribution was more concentrated in the perinuclear area. The distribution of hCAMΔtail was almost identical to that of hCAMΔ2nd Ig except that the protein was distributed to some dot-like structures in the cytoplasm. These results suggested that the 1st Ig domain is important for localization of hepaCAM on the plasma membrane. Secondly, the 2nd Ig and the cytoplasmic domain, although not critical for plasma membrane localization, play certain roles in fine tuning the intracellular distribution of hepaCAM.
Figure 4-12 Subcellular localization of wild type and truncated mutants of hepaCAM. Plasmids containing hepaCAM, hCAMΔ1stIg, hCAMΔ2ndIg, and hCAMΔtail were stably transfected into MCF7 cells. The subcellular localization of the proteins was revealed by immunofluorescence with anti-V5 antibody followed by confocal microscopy. Bars, 10 µm.
4.1.5.4 Association between integrin β1 and hepaCAM mutants

Next we determined if the physical association between hepaCAM and integrin β1 is dependent on certain domain(s). The cell lysate of MCF7 stable clones of WT hepaCAM, hCAMΔ1st Ig, hCAMΔ2nd Ig and hCAMΔtail was immunoprecipitated with anti-integrin β1 antibody followed by immunoblotting using anti-V5 antibody. The results showed that all mutants were able to be co-immunoprecipitated with integrin β1 (Fig. 4-13), indicating that deletion of any of the 1st Ig, the 2nd Ig, or the cytoplasmic domain does not affect the physical association between hepaCAM and integrin β1.

4.1.5.5 Association between hepaCAM and α subunits of integrin

We also attempted to identify the integrin α subunit that interacts with hepaCAM. Co-immunoprecipitation was performed using antibodies against the major types of α integrins expressed by MCF7 cells: integrin α2, α3, and αV. However, no interaction between hepaCAM and the α integrins tested could be detected (Fig. 4-14).

4.1.6 hepaCAM alters the expression and activity of integrin β1

4.1.6.1 hepaCAM up-regulates the protein expression of integrin β1

Subsequently, we examined if the expression of integrin β1 was affected by hepaCAM. Vector- or hepaCAM-transfected cells were spread on fibronectin for 0.5, 1.5 or 3 h followed by cell lysis and Western blot analysis. The results showed that the expression of total integrin β1 in hepaCAM-transfected cells was markedly increased at all time points when compared to the control cells (Fig. 4-15). RT-PCR
Figure 4-13 Association between integrin β1 and hepaCAM mutants. Cell lysate of MCF7 stable clones of wild type hepaCAM, hCAMΔ1st Ig, hCAMΔ2ndIg or hCAMΔtail was immunoprecipitated with anti-integrin β1 antibody followed by immunoblotting using anti-V5 antibody.
**Figure 4-14 Association between hepaCAM and α subunits of integrin.**
Cell lysate of hepaCAM(+) cells was immunoprecipitated with control IgGs or antibodies against integrin β1, α2, α3, or αV followed by immunoblotting using anti-V5 antibody.
Figure 4-15 hepaCAM up-regulates the protein expression of integrin β1. (A) hepaCAM(-) or hepaCAM(+) cells were plated on fibronectin. At the indicated time points, the cells were lysed and subjected to Western blot. An anti-integrin β1 antibody was used to detect the total protein level of integrin β1. GAPDH served as the loading control. (B) The bar chart shows the intensity of each protein band. Intensity of the band representing the vector transfectants at 0.5 h was considered as 1. Data represent means ± SD. *, P < 0.05.
was carried out to determine if the expression of integrin β1 was altered at the mRNA level. As shown in Figure 4-16, the mRNA level of integrin β1 in hepaCAM-transfected cells was comparable to that in the control cells at both 30 min and 3 h, suggesting that the hepaCAM-mediated integrin β1 up-regulation was not at transcription level.

### 4.1.6.2 hepaCAM increases the active form of integrin β1

To determine the activation of integrin β1, an antibody specific for the active form (high affinity state) of integrin β1 (9EG7) was used to perform immunoprecipitation. hepaCAM transfectants expressed a significantly higher level (approximately 1.5 fold) of active integrin β1 than the control cells at both 0.5 and 3 h time points (Fig.4-17).

### 4.1.6.3 hepaCAM increases the clustering of integrin β1

Activation of integrins is usually accompanied by lateral clustering (Li et al., 2003), which forms distinct patterns on the cell-ECM interface. To examine the effects of hepaCAM on integrin β1 clustering, the cells were spread on fibronectin-coated plate for 30 min followed by immunostaining with anti-V5 antibody. Consistent with the results in Figure 4-16, the immunofluorescence staining demonstrated robust clusters of integrin β1 in hepaCAM-transfected cells, whereas the integrin clusters observed in the control cells were considerably less (Fig. 4-18).
Figure 4-16 hepaCAM does not affect the mRNA level of integrin β1. (A) The mRNA level of integrin β1 in the cells was determined by semi-quantitative RT-PCR. (B) The bar chart shows the intensity of each RNA band. Intensity of the band representing the vector transfectants at 0.5 h was considered as 1. Data represent means ± SD.
Figure 4-17 hepaCAM increases the active form of integrin β1. (A) Lysate prepared from hepaCAM(-) or hepaCAM(+) cells was immunoprecipitated with an antibody specific for the active form of integrin β1 (clone 9EG7) followed by Western blot. IgG heavy chain bands served as a loading control. (B) The bar chart shows the intensity of each protein band. Intensity of the band representing the vector transfectants at 0.5 h was considered as 1. Data represent means ± SD. *, P < 0.05.
Figure 4-18 hepaCAM increases the clustering of integrin β1. The cells were spread on fibronectin coated surface for 30 min and stained with anti-integrin β1 antibody. The staining was examined by confocal microscopy. Bars, 10 μm.
4.1.6.4 Talin is not affected by hepaCAM

Talin has been established as the key molecule in the regulation of integrin activation (Calderwood et al., 2004). Western blot analysis was carried out to determine the expression level of talin in control and hepaCAM-transfected cells. Our results showed that the level of talin was not altered in hepaCAM-transfected cells compared to the control cells (Fig. 4-19 A). Furthermore, co-immunoprecipitation revealed no physical association between hepaCAM and talin (Fig. 4-19 B), even in the presence of DSP. These data suggested that the hepaCAM-mediated activation of integrin β1 is not likely to be talin-dependent.

4.1.7 hepaCAM affects focal contact assembly

4.1.7.1 hepaCAM is not localized at sites of focal contacts

Focal contacts are sites where integrin-mediated adhesions link to the actin cytoskeleton (Wozniak et al., 2004). These macromolecular assemblies serve to interconnect biochemical signaling between the ECM and the actin cytoskeleton. Therefore, it is important to investigate the influence of hepaCAM on focal contacts. To visualize if hepaCAM was localized at sites of focal contacts, hepaCAM-transfected cells were spread on fibronectin for 30 min and double-stained for hepaCAM and paxillin. Paxillin is a functionally important component of focal contacts and is often used as a marker for focal contacts in image studies. The immunofluorescence showed no co-localization of hepaCAM and paxillin (Fig. 4-20), indicating that hepaCAM is not localized at sites of focal contacts.
Figure 4-19 Talin is not affected by hepaCAM. (A) Cell lysate of hepaCAM(-) or hepaCAM (+) cells was subjected to Western blot with anti-talin antibody. (B) Lysate of hepaCAM(+) cells was immunoprecipitated with anti-talin antibody followed by immunoblotting with anti-V5 antibody.
**Figure 4-20 hepaCAM is not localized at sites of focal contacts.** hepaCAM transfectants were spread on fibronectin for 30 min and double-stained to detect hepaCAM (green) and paxillin (red). Bars, 10 μm.
4.1.7.2  hepaCAM regulates focal contact assembly in a temporal manner

4.1.7.2.1 hepaCAM changes the phosphorylation of FAK and paxillin

Next, we examined the effects of hepaCAM on the activity of two of the key components of focal contacts – focal adhesion kinase (FAK) and paxillin (Wozniak et al., 2004). These two proteins are activated through tyrosine phosphorylation (mainly Tyrosine 397 of FAK and Tyrosine 118 of paxillin) in response to integrin-ECM interactions. Western blot analysis showed that hepaCAM-transfected cells exhibited a higher total level of phospho-paxillin than the control cells at 30 min (Fig. 4-21). The level of phospho-FAK was not significantly changed. It is noteworthy that at 3 h, phospho-paxillin in hepaCAM transfectants appeared lower than the control cells, although the total level of paxillin remained higher than the control cells. There was also a decrease in phospho-FAK in hepaCAM-transfected cells compared to the control cells at 3 h.

4.1.7.2.2 hepaCAM increases the formation of focal adhesions at early stage of cell-ECM contact

The formation of focal contacts in vector- or hepaCAM-transfected cells was visualized by double-staining of paxillin and integrin β1. Focal contacts were more distinctly revealed by the co-localization of these two proteins. Immunofluorescence results showed that 30 min after plating on fibronectin, the control cells were not fully spread, and focal contacts were poorly formed (Fig. 4-22, a-c). In contrast, hepaCAM-transfected cells were well-spread, displaying abundant centrally located focal adhesions (FAs) as well as more peripherally located focal complexes (FXs) and nascent FAs as compared to the control cells at this time point (Fig. 4-22, d-f).
Figure 4-21 hepaCAM changes the phosphorylation of FAK and paxillin. (A) The protein levels of phosphorylated FAK (Y397), total FAK, phosphorylated paxillin (Y118) and total paxillin in hepaCAM(-) or hepaCAM(+) cells were examined by Western blot using respective antibodies. (B) The bar chart shows the relative intensity of each protein band. Intensity of the protein band representing control cells at 0.5 h was considered as 1. Data represent means ± SD. *, P < 0.05.
Figure 4-22 hepaCAM increases the formation of focal adhesions at early stage of cell-ECM contact. The cells were spread on fibronectin for 30 min and double stained using anti-integrin β1 (green) and anti-paxillin (red) antibodies. Bars, 10 μm. Arrow heads: focal adhesions.
4.1.7.2.3  *hepaCAM promotes the formation of less mature focal contacts at later stage of cell-ECM contact*

On the other hand, after spreading on fibronectin for 3 h, the control cells were fully spread and developed noticeable mature FAs (Fig. 4-23, g-i). Interestingly, FAs in hepaCAM-transfected cells were no longer observable. Instead, fine structures of less mature focal contacts (i.e. nascent FAs and FXs) at the periphery and the edge of the cells were prominent (Fig. 4-23, j-l), exhibiting features of migrating cells. Thus, while hepaCAM is not localized at sites of and not a component of focal contacts, it is capable of regulating focal contact assembly in a time dependent manner.

4.1.8  *hepaCAM alters the activity of Rho family GTPases*

Rho-family GTPases, including Rho, Rac and Cdc42, play important roles in integrin-mediated signaling. The classical model suggests that, among the three main Rho family GTPases, Rho regulates the assembly of actin stress fibers and FAs; Rac and Cdc42 are responsible for the formation of lamellipodia and filopodia, respectively (Nobes and Hall, 1995). The activity of GTPases in vector- and hepaCAM-transfected cells was examined by Rho/Rac/Cdc42 activation assay (Fig. 4-24). After plating on fibronectin for 30 min, hepaCAM-transfected cells expressed a significantly higher level of active Rho compared to that in the control cells, consistent with the observation that hepaCAM promoted cell spreading. However, the difference in active Rho was not obvious at 3 h. On the other hand, the level of active Rac in hepaCAM transfecants was significantly lower than the control cells at both time points. No significant difference in active Cdc42 was observed between control and hepaCAM-transfected cells.
Figure 4-23 hepCAM promotes the formation of less mature focal contact at later stage of cell-CM contact. The cells were spread on fibronectin for 3 h and double stained using anti-integrin β1 (green) and anti-paxillin (red) antibodies. Bars, 10 μm. Arrows: focal complexes.
Figure 4-24 hepaCAM alters the activity of Rho family GTPases. (A) hepaCAM(-) and hepaCAM(+) cells were spread on fibronectin for 0.5 or 3 h and evaluated for levels of active Rho by GST-Rhotekin pull-down, or for active Rac and Cdc42 by GST-PAK pull-down as described in Materials and Methods. (B) The bar charts show the relative intensity of each protein band. Intensity of protein band representing control cells at 0.5 h was considered as 1. Data represent means ± SD. *, P < 0.05.
4.1.9 hepaCAM alters the organization of the actin cytoskeleton

The actin cytoskeleton provides the mechanical strength driving cell adhesion and migration. To examine the effects of hepaCAM on the actin cytoskeleton dynamics, the cells were spread on fibronectin for 30 min or 3 h and stained with tetramethylrhodamine isothiocyanate (TRITC)-conjugated phalloidin to reveal F-actin. The difference in cytoskeletal organization between vector- and hepaCAM-transfected cells was obvious. While the control cells displayed disorganized actin structure (Fig. 4-25, left column), hepaCAM-transfected cells showed robust actin stress fibers, forming long bundles of filaments spanning the cytoplasm at 30 min (Fig. 4-25, right column, arrows). Moreover, highly compact meshworks of actin were found at the edge of hepaCAM-transfected cells at 3 h, forming large fan-shaped lamellipodia (arrow heads).

4.1.10 hepaCAM induces morphological changes of cells

The actin cytoskeleton is crucial for sustaining cell morphology. It is noteworthy that most of the control cells were polygonal, showing a typical epithelioid morphology. In contrast, a significantly higher percentage of hepaCAM-transfected cells displayed extended protrusions and polarized lamellipodia, resembling a more migratory, fibroblastoid phenotype. Quantification showed a 4-fold increase of cells exhibiting fibroblastoid-like morphology in hepaCAM-transfected cells (Fig. 4-26). Such morphology is well-associated with high motility (Sehgal, 2010).
Figure 4-25 hepaCAM alters the organization of the actin-cytoskeleton. hepaCAM(-) and hepaCAM(+) cells were spread on fibronectin for 0.5 or 3 h and stained with TRIC-conjugated phalloidin to reveal the organization of the actin cytoskeleton. Bars, 10 μm. Arrows: actin stress fibers. Arrowheads: lamellipodia.
Figure 4-26 hepaCAM induces morphological changes of MCF7 cells. Morphology of hepaCAM(-) or hepaCAM(+) cells after spreading on fibronectin for 3 h was photographed. Magnification, 320 ×. (B) Bar chart depicts the percentage of cells exhibiting fibroblastoid-like morphology (i.e. polarized cell shape with prominent lamillipodia at leading edge; arrowheads) quantified by analyzing more than 200 cells from 5 randomly selected fields. Data represent means ± SD. *, $P < 0.05$. 
4.2 Proteolytic cleavage of hepaCAM

4.2.1 Characteristics of hepaCAM cleavage

4.2.1.1 hepaCAM is proteolytically cleaved when re-expressed in MCF7 cells

The protein structure of hepaCAM has been described in the introduction (Section 1.5): it is a type-1 transmembrane Ig-like cell adhesion molecule of 416 amino acids, consisting of an extracellular domain, a trans-membrane domain, and a cytoplasmic domain (Fig. 4-27 A). The entire hepaCAM sequence was cloned into the eukaryotic expression vector pcDNA6B/V5-His, with the V5 epitope tagged at the cytoplasmic COOH-terminus of hepaCAM, allowing detection of hepaCAM protein with anti-V5 antibody.

The plasmid containing hepaCAM sequence was transfected into the human breast carcinoma cell line MCF7. The molecular mass of the V5-tagged hepaCAM was approximately 75 kD (Fig. 4-27 B, lane 2). In addition to the full-length protein of hepaCAM, a smaller band of approximately 25 kD was consistently observed, suggesting that hepaCAM undergoes a proteolytic cleavage.

4.2.1.2 Identification of a 25-kD species as a hepaCAM cleavage product

Since the 25-kD species was detected by anti-V5 antibody, this band should represent a fragment containing the cytoplasmic COOH-terminus. To verify that the 25-kD species was indeed derived from hepaCAM, a mutant with the truncation between
Figure 4-27 hepaCAM undergoes a proteolytic cleavage. (A) Sequences of the WT and truncated mutants of hepaCAM were cloned into the eukaryotic expression vector pcDNA6B/V5-His and transfected into the breast carcinoma cell line MCF7. EX, extracellular domain; TM, transmembrane domain; CT, cytoplasmic domain. □, signal peptide; ☐, V5 tag. (B) Protein expression of WT and mutant hepaCAM (hCAM-318 and hCAM-tail) in MCF7 cells was examined by Western blot using anti-V5 antibody.
amino acid 318 and 416 was constructed (Fig. 4-27 A). The mutant, designated hCAM-318, showed a molecular mass at about 60 kD and an additional band at about 10 kD (Fig. 4-27 B, lane 3), proving that the 25-kD species was the product of hepaCAM cleavage.

4.2.1.3 The cleavage product of hepaCAM contains mainly the cytoplasmic domain

To determine if the 25-kD fragment contains the cytoplasmic domain, we constructed another mutant of hepaCAM with the cytoplasmic domain only (residues 260-416), designated hCAM-tail (Figure 4-27 A). Interestingly, the cytoplasmic domain alone shared a similar molecular mass with the cleaved fragment (Fig. 4-27B, lane 4), suggesting that the 25-kD cleavage product contains mainly the cytoplasmic domain of hepaCAM.

4.2.1.4 The cleavage product of hepaCAM is membrane tethered

To determine whether the cleavage product of hepaCAM is dissociated from or tethered to the cell membrane, hepaCAM-transfected cells were fractionated into cytoplasmic and membrane portions. The purity of each fraction was confirmed by the detection of GAPDH (cytosolic marker) and E-cadherin (plasma membrane marker). As shown in Figure 4-28, a significant amount of the full-length hepaCAM was detected in the membrane fraction and a lower amount of the full-length protein appeared in the cytoplasmic fraction. However, the cleavage product of hepaCAM was predominantly detected in the membrane fraction, with almost undetectable
Figure 4-28 Subcellular distribution of hepaCAM cleavage product. hepaCAM transfectants were fractionated into cytosolic and membrane portions as described in materials and methods. The expression of full-length hepaCAM and the cleavage product was examined by Western blot. GAPDH served as a cytosolic marker while E-cadherin served as a membrane marker.
amount in the cytosolic fraction. These results indicated that the cleavage product of hepaCAM is membrane tethered.

4.2.2 Mechanisms involved in hepaCAM cleavage

4.2.2.1 Cleavage of hepaCAM is not dependent on cell density

Previously we have demonstrated that the subcellular localization of hepaCAM is dependent on cell density (Moh et al., 2005a, b). When the cell density is high, hepaCAM tends to localize on the plasma membrane, particularly at cell-cell boundaries. Whereas when the cells are sparse and well spread, hepaCAM proteins are less localized onto cell membranes but more into punctuate intracellular structures. Since the cleavage product of hepaCAM is membrane tethered, cell density, a factor that regulates the membranous distribution of the protein, is possible to play a role in the cleavage process.

To examine how cell density affects hepaCAM cleavage, hepaCAM-transfected cells were seeded at decreasing cell densities (100%, 50%, and 25% confluence). The cells were then subjected to Western blot analysis. As shown in Figure 4-29, hepaCAM displayed identical degrees of cleavage at three different cell densities, suggesting that unlike the subcellular localization, the cleavage of hepaCAM is independent of cell density.
Figure 4-29 hepaCAM cleavage is not dependent on cell density. hepaCAM-transfected cells were seeded at 100%, 50% or 25% density. After 24 h, the cells were lysed and subjected to Western blot with anti-V5 antibody. GAPDH served as the loading control.
4.2.2.2 The effects of the phorbol ester PMA on hepaCAM cleavage

4.2.2.2.1 PMA up-regulates hepaCAM protein expression

Phorbol esters have been reported to facilitate proteolytic cleavage of numerous cell surface proteins, such as E-cadherin (Maretzky et al., 2005) and CD44 (Okamoto et al., 1999). To determine if phorbol esters could promote hepaCAM cleavage, the effects of phorbol 12-myristate 13-acetate (PMA), one of the most commonly used phorbol esters, was examined. hepaCAM-transfected cells were treated with PMA for 15, 30, 60 or 120 min. As shown in Figure 4-30, PMA up-regulated both the full-length and the cleavage product of hepaCAM. Quantification of the intensities of the protein bands revealed that the ratio of the cleavage product versus the full-length of hepaCAM remained constant throughout the time courses, indicating that the degree of hepaCAM cleavage was not altered by PMA.

To test if prolonged treatment of PMA would have any effect on hepaCAM cleavage, we increased the course of PMA treatment up to 24 h. Cell lysate was harvested every 4 h and subjected to Western blot analysis. The cleavage of E-cadherin was examined as a positive control. Figure 4-31 shows that the cleavage product of E-cadherin was increased in a time dependent manner, while the expression level of full-length E-cadherin was hardly changed throughout the treatment, indicating a significant enhancement of the cleavage process of E-cadherin by PMA. PMA also significantly increased hepaCAM cleavage product time dependently (Fig 4-32), more prominently from 12 h onwards. However, unlike E-cadherin, the full-length of hepaCAM was up-regulated concurrently. Although the ratio of the cleavage product versus the full-length of hepaCAM increased consistently from 12 h after PMA treatment (Figure 4-32 B), such increase was probably due to the saturation of the protein bands.
Figure 4-30 Short-term of PMA treatment has no effect on hepaCAM cleavage. (A) hepaCAM-transfected cells were treated with PMA for 0, 15, 30, 60 or 120 min before lysis and Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of the molecular size markers are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart.
Figure 4-31 Effect of long-term treatment of PMA on E-cadherin. hepaCAM-transfected cells were treated with PMA for up to 24 h and cells were harvested every 4 h and subjected to Western blot with anti-E-cadhrin antibody. GAPDH served as the loading control. Positions of the molecular size markers are shown on the left of the panel.
Figure 4-32 Long term treatment of PMA up-regulates the protein expression of hepaCAM. (A) hepaCAM-transfected cells were treated with PMA for up to 24 h and cells were harvested every 4 h and subjected to Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of the molecular size markers are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
representing the full-length hepaCAM from 12 h onwards. Therefore, the data are not sufficient to support an increase of the cleavage process of hepaCAM. These results suggested that prolonged treatment of PMA further up-regulated the protein expression of hepaCAM compared to short-term treatment. The effect of PMA on hepaCAM cleavage is not obvious even when the treatment is prolonged.

### 4.2.2.2 PMA does not affect hepaCAM at mRNA level

Subsequently, semi-quantitative RT-PCR was carried out to examine if mRNA expression of hepaCAM was altered by PMA. It has been reported that mRNA of the intercellular cell adhesion molecule (I-CAM) can be up-regulated by PMA in renal cell carcinoma (Tanabe et al., 1997). I-CAM was therefore included as a positive control. The results revealed that the mRNA level of I-CAM reached peak 2 h after PMA treatment (Fig. 4-33, middle panel), whereas the mRNA level of hepaCAM remained unchanged up to 8 h after PMA treatment (Fig. 4-33, upper panel). It was noted that hepaCAM protein expression started increasing as early as 2 h upon PMA treatment. These results suggested that PMA induces an increase in hepaCAM protein expression but does not alter the transcription of hepaCAM.

### 4.2.2.3 PMA-induced protein up-regulation of hepaCAM is dependent on the PKC pathway

PMA is a known activator of PKC. To examine if PMA-enhanced hepaCAM cleavage was dependent on PKC activation, hepaCAM-transfected cells were pretreated with the specific PKC inhibitor GF109203X prior to the addition of PMA. As
Figure 4-33 PMA does not alter the mRNA level of hepaCAM. hepaCAM-transfected cells were treated with PMA for 0, 0.5, 1, 2, 4, or 8 h. The total RNA was extracted and RT-PCR was performed with primers specific for hepaCAM or I-CAM. GAPDH served as a loading control. (B) Intensities of RNA bands were measured and are represented in the bar chart (means ± S.D., n = 3).
demonstrated, pretreatment of GF109203X abolished the PMA-increased expression of hepaCAM (Fig. 4-34). This finding verified that PMA promoted hepaCAM protein expression through activation of PKC pathway.

4.2.2.3 PI3 kinase inhibitor suppresses hepaCAM cleavage

PI3 kinase (PI3K) is an important up-stream regulator of some PKC pathways (Morgensztern and McLeod, 2005). To examine if PI3K pathway is involved in hepaCAM cleavage, the cells were treated with a PI3K inhibitor wortmannin at indicated concentrations (Fig. 4-35). The results showed that the full-length of hepaCAM was not changed while the cleavage product was significantly decreased in a dose dependent manner, indicating a suppressed cleavage of hepaCAM. The data suggested that PI3 kinase plays a role in hepaCAM cleavage.

4.2.2.4 Calcium influx promotes hepaCAM cleavage

It is known that certain PKC isoforms are Ca\(^{2+}\) dependent (Bootman and Berridge, 1995). Elevation of intracellular Ca\(^{2+}\) level may activate PKC pathway and consequently regulate hepaCAM cleavage. The Ca\(^{2+}\) ionophore ionomycin is one of the most widely used reagent to induce calcium influx. The function of ionomycin is pH dependent. The optimal pH value for ionomycin to induce proteolysis was determined in E-cadherin, a known protein that its cleavage is regulated by calcium influx. Figure 4-36 shows that the cleavage of E-cadherin induced by ionomycin was enhanced as the pH value increased, reaching maximum at pH 8.5. Thus, subsequent experiments in this section were carried out at this pH.
Figure 4-34 The PMA-promoted hepaCAM protein expression is dependent on PKC. (A) hepaCAM-transfected cells were pre-treated with GF109203X for 2 h before treatment of PMA for 16 h. The cells were then lysed and subjected to Western blot with anti-V5 antibody. GAPDH served as loading control. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-35 Wortmannin treatment inhibits hepaCAM cleavage. (A) The cells were treated with wortmannin at indicated concentrations for 8 h and lysed for Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, $P < 0.05$. 
Figure 4-36 Ionomycin induced E-cadherin cleavage is pH dependent. The cells were treated with 1 µM ionomycin at indicated pH values for 15 min. The cells were then lysed for Western blot with anti-E-cadherin antibody. GAPDH served as the loading control. Positions of the molecular size marker are shown on the left of the panel.
To examine if Ca\(^{2+}\) influx could induce hepaCAM cleavage, the hepaCAM-transfected cells were treated with the Ca\(^{2+}\) ionophore ionomycin. The results showed that ionomycin caused a prominent accumulation of the cleavage product of hepaCAM, while the full-length of hepaCAM was correspondingly reduced (Fig. 4-37). Moreover, when the cells were co-treated with the Ca\(^{2+}\) chelator EDTA, the ionomycin-induced hepaCAM cleavage was diminished (Fig. 4-38, lane 4-6). These data indicated that the effects of ionomycin on hepaCAM cleavage was dependent on the influx of Ca\(^{2+}\) across the plasma membrane followed by the elevation of the intracellular Ca\(^{2+}\) level. In addition, the treatment of EDTA alone had no effect on the cleavage of hepaCAM (Fig. 4-38, lane 3), suggesting that the extracellular Ca\(^{2+}\) was not directly involved in the cleavage of hepaCAM.

One of the consequences of Ca\(^{2+}\) influx is the activation of protein kinase C (PKC) (Bootman and Berridge, 1995). To examine the role of PKC in ionomycin-induced hepaCAM cleavage, the cells were pretreated with the PKC inhibitor GF109203X followed by the treatment with ionomycin. As shown in Figure 4-39, GF109203X had no effect on ionomycin-stimulated cleavage of hepaCAM, suggesting that the Ca\(^{2+}\) influx-induced cleavage was independent of PKC pathway.

### 4.2.2.5 Proteasome inhibitors suppress hepaCAM cleavage

It is common that the cleavage product of a protein is not detectable due to immediate degradation mainly through the ubiquitin-proteasome system. For example, the cleavage product of CD44 is undetectable without the presence of proteasome inhibitor (Okamoto et al., 1999). To investigate if the 25-kD cleavage product of
Figure 4-37 Ionomycin treatment promotes hepaCAM cleavage. (A) The cells were treated with ionomycin at indicated concentrations for 15 min and lysed for Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-38 The effect of ionomycin on hepaCAM cleavage is through calcium influx. (A) The cells were treated with 1 µM ionomycin and/or EDTA at indicated concentrations for 15 min. The cells were then lysed for Western blot with anti-V5 antibody. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-39 Ionomycin-induced hepaCAM cleavage is independent of PKC pathway. (A) The cells were pre-treated with 5 µM G109203X followed by incubation with 1 µM ionomycin for 15 min. The cells were then lysed for Western blot with anti-V5 antibody. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart.
hepaCAM was subjected to proteasomal degradation, the cells were treated with a proteasome inhibitor MG132. Intriguingly, instead of being accumulated, the cleavage product of hepaCAM was significantly reduced after MG132 treatment in a time dependent manner (Fig. 4-40). This unexpected result indicated that the cleavage process of hepaCAM is rather distinct from that of other CAMs.

It is known that MG132 is not a specific inhibitor of proteasome. In addition to proteasome, MG132 has a broad spectrum of inhibitory effects on proteases, including β- and γ-secretase (Steinhilb et al., 2001; Murakami et al., 2003) and the cysteine proteases calpain-1 and cathepsin B (Steinhilb et al., 2001). To evaluate the involvement of proteasome, hepaCAM-transfected cells were treated with the specific proteasome inhibitor clasto-lactacystin β-lactone (mentioned as lactacystin in the subsequent text). Figure 4-41 shows that not only MG132 but also lactacystin significantly decreased the cleavage product with a slight increase of the full-length of hepaCAM. p53 is known to be ubiquitinated. Multiple bands at higher molecular weights representing ubiquitinated p53 products were observed after treatment with MG132 or lactacystin, confirming the effects of the two proteasome inhibitors. These results suggested that the proteasome is indeed involved in hepaCAM cleavage.

**4.2.2.6 β- and γ-secretases are not responsible for the MG132-induced suppression of hepaCAM cleavage**

Next, to evaluate the possible participation of other enzymes that were inhibited by MG132, the cells were treated with β-secretase inhibitor IV or the γ-secretase inhibitor DAPT. Interestingly, as shown in Figure 4-42, β-secretase did not inhibit
Figure 4–40 MG132 treatment inhibits hepaCAM cleavage. (A) The cells were treated with 5 µM MG132 for indicated time points and lysed for Western blot with anit-V5 antibody. GAPDH served as the loading control. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-41 The specific proteasome inhibitor Lactacystin inhibits hepaCAM cleavage. (A) The cells were treated with 5 µM MG132 or 2.5 µm lactacystin for 8 h and lysed for Western blot with ani-V5 antibody. GAPDH served as the loading control. p53 was included as the positive control of inhibition of ubiquitination. Positions of the molecular size marker are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, $P < 0.05$. 
Figure 4-42 The effect of β- and γ-secretase inhibitors on hepaCAM cleavage. (A) The cells were treated with MG132 (5 µM), β-secretase inhibitor IV (10 µM), DAPT (10 µM), or pepstatin A methyl ester (20 µM) for 16 h. Cell lysates were analyzed by Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of the molecular size markers are shown on the left of the panel. (B) The ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05. β–sec IV: β-secretase inhibitor IV; PAME: pepstatin A methyl ester.
the cleavage of hepaCAM. Conversely, it resulted in an apparent increase of hepaCAM cleavage product. In addition, DAPT had no effect on hepaCAM cleavage. These results indicated that β- and γ-secretase were not responsible for the MG132-induced suppression of hepaCAM cleavage.

4.2.2.7 Involvement of cysteine proteases in hepaCAM cleavage

4.2.2.7.1 Calpain-1 is involved in hepaCAM cleavage

As MG132 also inhibits cysteine proteases calpain and cathepsin, we finally investigated the involvement of these enzymes in hepaCAM cleavage. Two calpain inhibitors MDL28170 and calpeptin were used to examine the effects of calpain inhibition on hepaCAM cleavage. The results showed that both inhibitors significantly reduced the cleavage product and accumulated the full-length of hepaCAM (Figure 4-43 and 44). As MCF7 cells are deficient of calpain-2 (Wu et al., 2006), we assayed for the activation status of calpain-1. In the inactive condition, calpain-1 exists as pro-enzyme heterodimer of 80-29 kD, and following Ca$^{2+}$ binding undergoes autolysis to an active 78-18 kD heterodimer (Wang, 2000). Western blot analysis revealed the presence of both 78- and 80-kD species of calpain-1 large subunit in untreated cells (Fig. 4-45, lane 1), indicating a basal activity of this enzyme. Following treatment of MDL28170 and calpeptin, the autolysis of calpain-1 was prevented, and a clear accumulation of the 80-kD large subunit was observed (Fig. 4-45, lanes 2 & 3). These results provided strong evidence that calpain-1 may be involved in hepaCAM cleavage.
Figure 4-43 The effect of MDL28170 on hepaCAM cleavage. (A) The cells were treated with 10 μM MDL28170 for indicated time points and lysed for Western blot with anti-V5 antibody. GAPDH served as loading control. Positions of the molecular size markers are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, $P < 0.05$. 
Figure 4-44 The effect of calpeptin on hepaCAM cleavage. (A) The cells were treated with 10 μM MDL28170 for indicated time points and lysed for Western blot with anti-V5 antibody. GAPDH served as loading control. Positions of the molecular size markers are shown on the left of the panel. (B) Intensities of protein bands were measured, and the ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-45 Caplain-1 is inhibited by MDL28170 and calpeptin. The cells were treated with 100 μM MDL28170 or 10 μM calpeptin for 8 h and lysed for Western blot with anti-calpain-1 antibody. MDL: MDL28170; CPT: calpeptin.
We have demonstrated that calcium influx promotes hepaCAM cleavage. Since calpain is a calcium dependent enzyme, it is interesting to study if calpain plays any role in calcium influx-enhanced hepaCAM cleavage. hepaCAM-transfected cells were pretreated with the calpain inhibitor MDL28170 for 2 h followed by the treatment of ionomycin for 15 min. As shown in Figure 4-46, MDL28170 significantly suppressed ionomycin-triggered increase of hepaCAM cleavage, suggesting that the effects of calcium influx on hepaCAM cleavage is partly contributed by calpain-1.

4.2.2.7.2 Cathepsin B is involved in hepaCAM cleavage

The cysteine proteases cathepsin B and L have been reported to be inhibited by MG132 (Malen et al., 2007; Goulet et al., 2004). We showed that two cathepsin inhibitors Z-FF-FMK (cathepsin B and L inhibitor) and CA074-Me (cathepsin B inhibitor) efficiently decreased hepaCAM cleavage (Fig. 4-47). Taken together, these data suggested that calpain-1 and cathepsin B, members of cysteine proteases, were involved in the cleavage of hepaCAM.

4.2.3 The cytoplasmic domain plays important roles in hepaCAM functions

4.2.3.1 The cytoplasmic domain is essential for hepaCAM-mediated cell-ECM adhesion

We have demonstrated that the cleavage product of hepaCAM contains mainly the cytoplasmic domain. To investigate if the remaining moiety after cleavage has any functional role, a mutant of hepaCAM with the truncation of the entire cytoplasmic domain (residues 264-416) was constructed (Fig. 4-48A). Designated as hCAM\Delta tail,
Figure 4-46 Calpain inhibitor inhibits ionomycin-enhanced hepaCAM cleavage. The cells were pre-treated with 100 μM MDL28170 before treatment with 1 μM ionomycin for 15 min. The cells were then lysed for Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of molecular size markers are shown on the left of the panel.
Figure 4-47 Effect of cathepsin-B inhibitors on hepaCAM cleavage. (A) The cells were treated with 10 μM Z-FF-FMK or 10 μM CA074-Me for 8 h and lysed for Western blot with anti-V5 antibody. GAPDH served as the loading control. Positions of molecular size markers are shown on the left of the panel. (B) The ratios of cleavage product against full-length of hepaCAM (means ± S.D., n = 3) are represented in the bar chart. *, P < 0.05.
Figure 4-48 Generation of cytoplasmic domain-deleted mutant of hepaCAM.
(A) Schematic representation of WT hepaCAM and hCAMΔtail. EX, extracellular domain; TM, transmembrane domain; CT, cytoplasmic domain. ☐, signal peptide; ☐, V5 tag. (B) Protein expression of WT hepaCAM and hCAMΔtail in MCF7 cells was examined by Western blot with anti-V5 antibody.
this mutant was also used in the study of hepaCAM-integrin β1 interaction. hCAMΔtail was stably expressed in MCF7 cells (Fig. 4-48B). The adhesive property of MCF7 cells expressing pcDNA6B vector, WT hepaCAM or hCAMΔtail on fibronectin was evaluated through cell adhesion assay. Compared to control cells 15% and 52%, the spreading of hepaCAM-transfected cells was 35% and 74% \( (P < 0.05) \) and hCAMΔtail-transfected cells 11% and 47% \( (P > 0.05) \), at 30 min and 2 h of incubation respectively (Fig. 4-49). These results suggested that when the cytoplasmic domain is truncated, hepaCAM loses the ability to promote cell-ECM adhesion.

**4.2.3.2 The cytoplasmic domain is critical for hepaCAM-mediated cell migration**

The motility of MCF7 cells expressing pcDNA6B vector, WT hepaCAM or hCAMΔtail was assessed by the wound closure assay. Confluent monolayer of cells were wounded and allowed to heal. After 24 h of incubation, the wound closure was 56% by hepaCAM-transfected cells, whereas 32% by hCAMΔtail transfectants, and 25% by the control cells.

After 48 h, the wound closure reached 86% by hepaCAM transfectants, 59% by hCAMΔtail transfectants and 51% by the control cells (Fig. 4-50). hepaCAM transfectants displayed the fastest wound closure \( (P < 0.05) \) and the closure rate of hCAMΔtail transfectants was comparable \( (P > 0.05) \) to that of the control. Moreover, at 3 h, hepaCAM-transfected cells at the edge of the wound showed noticeable cell protrusions (Fig. 4-50, middle panel, arrowheads), which marked the start of cell migration. When the cytoplasmic domain was deleted, such protrusions were not
Figure 4-49 Effects of cytoplasmic domain truncation on hepaCAM-mediated cell-ECM adhesion. (A) Cells stably expressing pcDNA6B vector, hepaCAM or hCAMΔtail were spread on fibronectin-coated surface. After 30 min or 2h, the morphology of cells was observed and microscopic pictures were taken. 200 × magnification. (B) Cells exhibiting spread morphology was counted in five randomly selected fields and the percentages were calculated and presented in the bar chart (means ± S.D., n = 5). *, P < 0.05.
Figure 4-50 Effects of cytoplasmic domain truncation on hepaCAM-mediated cell motility. (A) Confluent monolayers of cells transfected with pcDNA6B vector, WT hepaCAM or hCAMΔtail were scratched and allowed to grow. Microscopic pictures were taken after 24 h and 48 h. 100× magnification. (B) Percentages of wound closure at 48 h are represented in the bar chart. (means ± S.D., n = 3). *, P < 0.05.
observed (Fig. 4-51, right panel), similar to the control cells (Fig. 4-51, left panel). These results indicated that when the cytoplasmic domain is cleaved, hepaCAM is not able to enhance cell motility.

4.2.3.3 The cytoplasmic domain plays a key role in the growth inhibitory effects of hepaCAM

4.2.3.3.1 Colony formation

To study the role of the cytoplasmic domain in cell growth mediated by hepaCAM, MCF7 cells were transfected with either WT hepaCAM or hCAMΔtail. Cells transfected with the pcDNA6B vector served as the control. After 12 days of antibiotic selection, the colonies formed were stained with crystal violet for observation (Fig. 4-52). Compared to the vector control, expression of hepaCAM strongly reduced both the number and the size of cell colonies. The expression of hCAMΔtail less significantly inhibited colony formation.

4.2.3.3.2 Cell proliferation

Furthermore, the growth rate of cells transfected with pcDNA6B control, WT hepaCAM or hCAMΔtail was determined by MTT cell proliferation assay (Fig. 4-53). Compared to that of the control cells, the growth rate of hepaCAM transfectants was significantly slower ($P < 0.05$) on day 5. However, hCAMΔtail did not clearly inhibit the proliferation of MCF7 cells. Together with the colony formation results, these data implied that the inhibitory effect of hepaCAM on cell growth is diminished when the cytoplasmic domain is truncated.
Figure 4-51 hepaCAM-transfected cells displayed protrusions during migration.
In the same wound closure assay as in Fig. 4-49, microscopic photos of cell morphology at the edge of wound were taken 3 h after the scratches were made. Arrowheads: cell protrusions. 320 × magnification.
Figure 4-52 The effect of cytoplasmic truncation on the hepaCAM-mediated suppression on colony formation. (A) MCF7 cells were transfected with pcDNA6B vector, hepaCAM or hCAMΔtail and selected in 10 μg/ml blasticidin for 12 days. The colonies formed were stained with 1% crystal violet (upper row). Microscopic photos of the average cell density in a stained colony were taken at 100 × magnification (lower row). (B) The number and size of colonies (means ± S.D., n = 5) are represented in the bar charts. *, P < 0.05.
Figure 4-53 The effect of cytoplasmic domain truncation on the hepaCAM-mediated inhibition on cell proliferation. Growth rate of cells transfected with pcDNA6B vector, hepaCAM or hCAMΔtail was monitored by MTT assay for 5 days. Data represent means ± SD of three experiments performed in triplicates. *, P < 0.05.
4.2.3.3 Cell cycle

To study if the antiproliferative effect of hepaCAM was a consequence of cell cycle arrest, flow cytometry was carried out to determine the cell cycle distribution of the cells. Intriguingly, hepaCAM transfectants displayed a unique cell cycle profile in that 83% of the cell population was accumulated in the G2/M phase (Fig. 4-54), indicating a significant G2/M cell cycle arrest. However, MCF7 cells expressing hCAMΔtail displayed a cell cycle profile similar to that of the control cells, suggesting that the cytoplasmic domain was critical for the regulation of cell cycle by hepaCAM.
Figure 4-54 The effects of cytoplasmic domain truncation on the hepaCAM-mediated cell cycle regulation. Cell cycle distribution of cells transfected with pcDNA6B vector, hepaCAM or hCAM\(\Delta\)tail was determined by flow cytometry. Percentages of cells in each phase (means ± S. D., n = 3) are represented in the bar chart. ***, \(P < 0.001\).
CHAPTER 5  DISCUSSION

5.1  Connection between hepaCAM and the integrin-dependent signaling

5.1.1  hepaCAM accelerates initial cell attachment

Cell adhesion is generally comprised of four steps: cell attachment to ECM substrate, cell spreading, focal contact assembly and cytoskeletal re-organization (Adams, 1997). The first step - initial cell attachment - is distinct from the following processes as this step is entirely dependent on the direct interaction between the adhesion molecules - in most cases integrins - and the ECM proteins. An increase in initial cell attachment is usually associated with an enhanced integrin affinity. The data on cell attachment provided the evidence that hepaCAM changes the affinity of the integrin(s) which mediate the cell attachment to the fibronectin substrate.

5.1.2  hepaCAM increases cell spreading

Following cell attachment to an ECM substrate is cell spreading, which involves more complicated signaling events leading to focal contact assembly and cytoskeletal re-organization (Adams, 1997). The cell spreading results suggested that besides altering the activity of integrins, hepaCAM also affects the downstream signaling following cell-ECM contact.

5.1.3  hepaCAM promotes both the velocity and directionality of cell migration

Cell migration can be conceptualized as a cyclic process. The first step is to extend cell protrusions in the direction of migration. The protrusions can be large, broad lamellipodia or spike-like filopodia. These structures are driven by actin polymerization and stabilized by adherence to the ECM substrate via various CAMs.
These adhesions serve as traction sites during migration as the cell moves forward over them. They are disassembled at the cell rear to allow the cell to detach (Huttenlocher et al., 1995). Thus, modulation in cell adhesion would result in changes in migration. Time-lapse microscopy enabled us to observe the behaviors of individual cells during migration and showed an overall more mobile phenotype of hepaCAM-transfected cells. The enhanced velocity of hepaCAM-transfected cells was consistent with our previous results on wound healing (Moh et al., 2005a, b, 2008). In addition, hepaCAM also enhanced the directionality of migration. Conceptually, directional cell migration has two sources: intrinsic cell directionality and external regulation. Intrinsic directionality is observed when cells respond to a non-directional motogenic signal, such as the uniform application of platelet-derived growth factor (PDGF) that triggers the basic motility machinery in the absence of any external guiding factor. Random migration occurs when a cell has low intrinsic directionality. If the motogenic stimulus is presented as an external gradient or with another external guidance cue, the cell then exhibits induced directionality (Petrie et al., 2009). The hepaCAM-induced directionality was intrinsic as no triggers such as chemical gradient or chemoattractant were included in the study.

5.1.4 Physical association between hepaCAM and integrin β1

We showed that hepaCAM physically associated with integrin β1 through cis conformation. Whether this interaction is direct or indirect is not clear. The in vitro translated hepaCAM and integrin β1 were not co-immunoprecipitated, suggesting an indirect interaction. However, it is noteworthy that the translated hepaCAM had a molecular size of about 50 kD, smaller than the 75 kD size of the exogenously expressed hepaCAM in MCF7 cells. This is probably due to the lack of
posttranslational modification of hepaCAM. It has been shown that different glycosylation on the extracellular domain of hepaCAM leads to distinct profiles of interacting partners (Gaudry et al., 2008), suggesting the importance of posttranslational modification in protein-protein interaction; therefore the results from the *in vitro* translated proteins may not reflect the true property of hepaCAM. The interaction between integrin \( \beta1 \) and native hepaCAM remains to be further investigated.

5.1.5 **Truncated mutants of hepaCAM**

When exogenously expressed in MCF7 cells, WT hepaCAM, hCAM\( \Delta1^{st} \) Ig, hCAM\( \Delta2^{nd} \) Ig and hCAM\( \Delta \)tail were expressed at different protein levels that hCAM\( \Delta \)tail displayed the highest expression and hCAM\( \Delta1^{st} \) Ig showed the lowest expression. Because the protein expression was examined in stable cell clones, the difference should not be derived from different transfection efficiencies of each plasmid but rather a reflection of the role of each domain in regulation of hepaCAM protein expression. Several clones of each mutant were examined to ensure that the difference was not derived from different sites of chromosomal integration. The 1\(^{st} \) Ig domain seems to be important to the expression and/or stabilization of hepaCAM protein as deletion of this domain results in significantly reduced protein level. Whereas the cytoplasmic domain appears to be inhibitory to the expression and/or stabilization of hepaCAM since hCAM\( \Delta \)tail has an increased expression compared to the WT. In addition, both hCAM\( \Delta2^{nd} \) Ig and hCAM\( \Delta \)tail showed double bands with at least one band of a smeared shape, while hCAM\( \Delta1^{st} \) Ig had a distinct single band. The smearing effect of a protein band is usually due to posttranslational modification such as glycosylation (Vina-Vilaseca et al., 2011). We have previously shown that
hepaCAM undergoes N-linked glycosylation (Moh et al., 2005b). In another experiment, when deglycosylated with PNGase, all three truncated mutants shifted to a smaller molecular size and displayed single bands, suggesting that N-linked glycosylation is retained in all truncated mutants, thus glycosylation occurs on both 1st and 2nd Ig domains.

Subcellular localization of the truncated mutants showed that, in line with the Western blot results, hCAMΔ1st Ig showed a relatively low staining intensity compared to the WT and other mutants. In addition, hCAMΔ1st Ig was the only mutant that lost the localization on the plasma membrane. Thus the 1st Ig domain is not only important for maintaining the level of protein expression, but also critical for the localization of hepaCAM on the plasma membrane. It has been demonstrated that the subcellular localization of SIRT1 is changed by increased protein stability (Byles et al., 2011), and the subcellular localization of E2F-1 protein affects its stability (Ivanova et al., 2006). It is possible that the expression level of hepaCAM and the subcellular localization are also interrelated.

5.1.6 Integrin β1 associates with all truncated mutants of hepaCAM

The immunoprecipitation data showed that all truncated mutants tested, including hCAMΔ1st Ig, hCAMΔ2nd Ig and hCAMΔtail, were able to co-immunoprecipitate with integrin β1, suggesting that none of the domains – the 1st Ig, the 2nd Ig and the cytoplasmic domain - is indispensable for the interaction between hepaCAM and integrin β1. It is possible that multiple binding sites are involved in the interaction and deletion of any single domain does not affect the overall interaction between the two
proteins. Another possibility is that hepaCAM indirectly interacts with integrin β1 through other proteins. If the truncated mutant remains associated with other binding partners, the association with integrin β1 would be retained.

It was mentioned that the interaction between WT hepaCAM and integrin β1 could only be detected when the cells were treated with the crosslinking agent DSP, indicating a weak interaction. Similarly, DSP was required to co-immunoprecipitate integrin β1 with hCAMΔ1st Ig and hCAMΔ2ndIg. However, the co-immunoprecipitation of integrin β1 and hCAMΔtail was easily detected without crosslinking, suggesting that the interaction between integrin β1 and hCAMΔtail is rather firm. Therefore, the presence of the cytoplasmic domain of hepaCAM may destabilize the interaction with integrin β1. Alternatively, the high expression of hCAMΔtail may contribute to the more stable interaction with integrin β1.

Previously we have shown that only intact hepaCAM is able to interact with F-actin. The truncated mutants - hCAMΔ1st Ig, hCAMΔ2ndIg and hCAMΔtail - all lost this interaction (Moh et al., 2008). It is well known that integrins link with the actin cytoskeleton via complex adaptor proteins (Blystone, 2004). Since deletion of any of the domains would lead to the loss of hepaCAM-actin interaction, it is not likely that the interaction between hepaCAM and integrin β1 is through actin.
5.1.7 The interacting integrin α subunit is not identified

Integrins are heterodimeric receptors composed of α and β subunits. We have identified integrin β1 as an interacting partner of hepaCAM; in theory, hepaCAM should also be associated with an α subunit that pairs with integrin β1 to form the functional heterodimer. However, the major integrin α subunits in MCF7 cells - α2, α3, and αV- were not shown to interact with hepaCAM. There may be several reasons: experimentally, the antibodies used might not be suitable for immunoprecipitation; or the condition of immunoprecipitation was not optimized. Alternatively, it is possible that the interacting α subunit is not among the ones we have tested. It might be a less represented integrin α that is expressed at a low level in MCF7 cells.

5.1.8 hepaCAM up-regulates the protein expression and total activity of integrin β1

Integrins remain inactive under normal condition and are converted to an active form upon ligand binding, i.e. binding to ECM substrates. The activation process drastically enhances the affinity of integrins, leading to cell attachment and spreading (Calderwood, 2004). An accelerated initial cell attachment is usually associated with increased affinity of surface integrins towards the binding substrate. Our data demonstrated that the expression of integrin β1, more importantly the active form (i.e. with high affinity towards ECM), was significantly up-regulated in hepaCAM-transfected cells. This finding is particularly important as it provides the basis for explaining the rapid initial attachment of hepaCAM-transfected cells.
There are two possibilities that the total activity of integrin β1 is increased: 1) through the increase of the overall protein expression; or 2) by affecting regulators of integrin activation. The first possibility was supported by the experimental data that the increase of total protein expression of integrin β1 in hepaCAM-transfected cells was almost proportional to the increase of the active form. The increase of integrin β1 was not at the mRNA level, suggesting that hepaCAM probably affects posttranscriptional regulation or translation of integrin β1. How hepaCAM alters these processes remain to be further explored. The second possibility was tested by examining the effects of hepaCAM on talin. Talin has long been considered as the sole regulator of integrin activation (Moser et al., 2009). It directly binds to the cytoplasmic domain of integrin and causes conformational changes that lead to increased affinity. However, neither the expression of talin was changed by hepaCAM nor was there any interaction between talin and hepaCAM. Thus the increased active form of integrin β1 by hepaCAM is likely to be independent of talin. Nevertheless, kindlins have recently been emerged as a class of novel regulators of integrin activity (Moser et al., 2008). The interaction between hepaCAM and kindlins is worth further investigation.

5.1.9 hepaCAM regulates focal contact assembly

Following cell attachment to an ECM substrate is cell spreading, which involves focal contact assembly and cytoskeletal re-organization (Adams, 1997). Focal contacts are complex structures consisting of multiple protein complexes that transduce bi-directional mechanical and chemical signals between extracellular and intracellular compartments (Bershadsky et al., 2003; Chen et al., 2004; Geiger et al., 2001). The stability and size of focal contacts increase in a complicated process of maturation.
Nascent FAs (focal points) and FXs are newly formed focal contacts that are distributed at the extreme edge or periphery of the cells; whereas FAs are mature, large and stable focal contacts that are predominantly located at central area of the cells (Wozniak et al., 2004). Our results showed that hepaCAM was not localized at the sites of focal contacts, therefore not a component of the complex focal contact structures. However, the assembly of focal contacts in hepaCAM-transfected cells at different time points differed significantly from that of the control, suggesting a regulatory role of hepaCAM in focal contact assembly. Many cell types exhibit a biphasic relationship between cell migration speed and substratum adhesiveness (Huttenlocher et al., 1996; Gobin and West, 2002), with maximum migration rates at intermediate adhesiveness where cells can both efficiently form adhesions at the cell front and release adhesive contacts at the cell rear (DiMilla et al., 1993; Huttenlocher et al., 1995). It has been postulated that molecules strengthening cell-ECM adhesion would result in decreased cell migration due to “adhesive brake” (Shi et al., 2007). However, the optimal adhesiveness for cell migration is strictly dependent on cell type and the ECM substratum. A striking feature of hepaCAM is that it exhibits temporal regulation on focal contact assembly. At early time points of cell-ECM contact, hepaCAM promoted the formation of stable FAs allowing cells to adhere to the substratum efficiently. However at later time points, while the control cells developed mature FAs and establish stable cell-ECM adhesion, focal contacts in hepaCAM-expressing cells were predominantly localized at cell periphery, where less mature focal contacts (i.e. nascent FAs and FXs) with high turnover were formed, favoring cell migration. Therefore, the increased cell velocity of hepaCAM-transfected cells during migration could be due to the subsequent decreased cell-ECM adhesiveness as a result of the regulation of focal contact assembly.
Moreover, the Western blot results of phosphorylated FAK and paxillin were in line with the immunofluorescence images. The data showed an overall increase of focal contact assembly in hepaCAM-transfected cells compared to the control cells at early time points and decrease at later time points. However, the difference, although statistically significant (~10%, $P < 0.05$), was not as drastic as what was observed from the confocal photographs. This is probably due to the changes in focal contact assembly often occurring at confined areas, e.g. the periphery of cells. In other word, it is rather a “local” than a “global” effect; therefore only a relatively small fraction of focal contact proteins are involved. When performing Western blot analysis, proteins from the whole cell lysate are examined, and the difference between small fractions of proteins would be masked.

5.1.10 hepaCAM alters the activity of Rho and Rac

The Rho family of small GTPases play crucial roles in various cellular behaviors including cell adhesion, migration, and morphology (Evers et al., 2000). The classic model of Rho family GTPases assigns specific roles to each of the members, e.g. Rho regulates the assembly of actin stress fibers and FAs, and is important for cell spreading and contractility; Rac and Cdc42 are responsible for the formation of lamellipodia and filopodia, respectively, and are crucial for cell polarization and migration (Nobes and Hall, 1995). However, many lines of evidence support an intrinsically more complicated picture of Rho family GTPases signaling, which sometimes contradicts with the classic model. For example, in contrast to the clear effects of the overexpression of double negative (DN) mutants, cells in which Rac1 (Wheeler et al., 2006) or Cdc42 (Czuchra et al., 2005) have been genetically ablated
can still protrude lamellipodia and produce filopodia, and only display very mild defects in cell migration. This suggests that the contributions of Rac1 and Cdc42 to the process of cell migration are much more subtle than we initially thought. Another example is the finding that RhoA is linked not only to the generation of contractility, but also to the regulation of microtubule stabilization at the leading edge during directional cell migration (Bartolini and Gundersen, 2009; Palazzo et al., 2001). Rather than involving Rho kinase, this specific RhoA function requires stabilization of microtubules through the effector mDia. RhoA therefore most probably performs different functions at the front and at the back of migrating cells, undoubtedly through selective interactions with different downstream effectors. A recent study using advanced imaging techniques provide new insight into the inter-relationships of Rho family GTPases: multiple Rho GTPase signaling modules crosstalk in a spatial and temporal manner to regulate specific morphogenetic events of the cells (Pertz, 2010).

Our data showed that Rho activity was increased by hepaCAM at an early stage of cell adhesion, consistent with the increased cell spreading and actin stress fiber formation. Furthermore, Rac activity was reduced in hepaCAM-transfected cells at both stages. This finding is particularly interesting. As shown in the results with cell morphologies, hepaCAM-transfected cells often display prominent protrusions and large lamellipodia, which in the classic model are usually associated with a higher activity of Rac. Thus our results were contradictory to the classic conception. However, it has been recently reported that Rac is closely related to the directionality of intrinsic cell migration. Even a very small decrease in Rac activity can switch random migration to directional (Pankov et al., 2005). Therefore, the reduced Rac activity in hepaCAM-transfected cells may underlie the directional mode of migration.
induced by hepaCAM. The mechanism of how hepaCAM decreases the activity of Rac is unclear. Immunoprecipitation has been performed and no interaction between hepaCAM and Rac is identified. Whether hepaCAM affects the factors that control the GDP-GTP cycle, such as GEFs and GAPs, is worth further investigation.

5.1.11 hepaCAM alters the organization of the actin cytoskeleton and cell morphology

One of the most striking features of hepaCAM-transfected cells is the differed cell morphology. Cell morphology/shape is determined by the cytoskeleton, which includes three subclasses: microfilaments (actin), microtubules and intermediate filaments. Among the three, microfilaments are the most dynamic and play a central role in cell shape and motility (Pollard and Cooper, 2009). They give mechanical support to cells and hardwire the cytoplasm with the surroundings to support signal transduction. Different cell types have different cell shape and cytoskeleton structures. The majority of MCF7 cells display a disorganized structure of actin cytoskeleton (Li et al., 2008). However the expression of hepaCAM induced the formation of actin stress fibers at early time points and lamellpodia at later time points of cell-ECM contact. Previously we reported the direct interaction between hepaCAM and F-actin (Moh et al., 2009). Working in a complex with integrin β1 and actin, hepaCAM may serve as a modulator that conveys and relays extracellular signals to the intracellular cytoskeletal machinery. Whether the altered organization of the actin cytoskeleton is the consequence of altered integrin activity or induced by hepaCAM directly needs to be further explored. Furthermore, decreased Rac activity has also been documented to be associated with a more fibroblastoid and migratory phenotype (Evers et al., 2000).
Thus, our data suggested that hepaCAM, possibly in collaboration with other signaling components, regulates Rho- and Rac-mediated effects on the actin cytoskeleton, leading to the observed motile cell morphology with extended protrusions and polarized lamellipodia. The effects of hepaCAM on microtubules and intermediate filaments, the other two types of cytoskeleton that support cell shape, will be explored in future studies.

5.1.12 hepaCAM, a tumor suppressor or oncogene?

This study uncovers a novel connection between hepaCAM and integrin-dependent signaling and provides the molecular basis for the hepaCAM-mediated cell-ECM interaction. On the other hand, it also raises some intriguing questions. Previously we have demonstrated that hepaCAM is commonly suppressed in human cancers and exhibits suppressive effects on cell growth, showing properties of a tumor suppressor (Moh et al., 2005b). Here we show that hepaCAM time dependently promotes both cell-ECM adhesion and migration, which under most circumstances have opposing effects on cancer. While firm adhesion would lower the rate of extravasations and prevent cancer cell invasion, increased cell motility would promote metastasis. Moreover, as a putative tumor suppressor, how can the inhibitory effect of hepaCAM on cell growth be integrated with the promoting role in cell motility?

Many CAMs function as tumor suppressors, e.g. E-cadherin (Watabe et al., 1994), CADM1 (Yageta et al., 2002; Heller et al., 2007), and integrin α7 (Ren et al., 2007). When introduced into cancer cells, these molecules not only suppress cell growth but
also retard cell motility. However, evidence is currently emerging to support the
dictory roles of CAMs that both inhibit cell growth and promote cell motility
when restored in cancer cells. In addition to hepaCAM, the Ig superfamily
carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1) is
implicated in functioning as a tumor suppressor and as a metastasis promoter. The
characteristics and functions of CEACAM1 have been demonstrated in individual
reports. CEACAM1 is frequently downregulated or dysregulated in multiple human
tumors (Riethdorf et al., 1997; Neumaier et al., 1993), and is capable of suppressing
cell growth and inducing apoptosis (Nittka et al., 2008). Ebrahimnejad et al. (2004)
demonstrated that exogenous expression of CEACAM1 enhances melanoma cell
invasion and migration; and this enhanced motility can be reverted by anti-
CEACAM1 antibodies. The ability of CEACAM1 to co-stimulate tumor suppression
and invasion was finally established by Liu et al. (2007) in restricting thyroid cancer
growth but promoting invasiveness. Introduction of CEACAM1 into CEACAM1-
deficient thyroid cancer cells results in G1/S phase cell cycle arrest accompanied by
elevated p21 expression and diminished Rb phosphorylation. Overexpression of
CEACAM1 also increases cell-ECM adhesion but promotes cell migration and tumor
invasiveness. In xenografted mice, CEACAM1 expression results in reduced tumor
growth and increased tumor invasiveness. Conversely, silencing of endogenous
CEACAM1 accelerates tumor growth and suppresses invasiveness (Li et al., 2007).

The paradox of the contradictory roles that hepaCAM and CEACAM1 possess on
cancer development has not been resolved. The expression levels, the types and stages
of tumors, as well as other intracellular or extracellular cues could be important factors
that affect their functions. In a previous study, we showed that hepaCAM inhibits the
growth rate and suppresses wound healing of the glioblastoma U373 cells (Lee et al., 2009), suggesting that hepaCAM may have different functions in different cell types, probably due to distinct interacting partners and local environment. In addition, temporal regulation of protein expression may be a plausible hypothesis to explain the paradox. It has been shown that the expression of CEACAM1 is spatially and temporally regulated during different stages of malignancies (Riethdorf et al., 1997; Liu et al., 2007). A model that covers all stages of cancer development would be helpful to reveal if the expression of hepaCAM is differentially regulated during cancer progression.
5.2 The proteolytic cleavage of hepaCAM

5.2.1 hepaCAM undergoes a proteolytic cleavage when expressed in MCF7 cells

In this study, we demonstrated for the first time that hepaCAM undergoes a proteolytic cleavage when exogenously expressed in MCF7 cells. It would be valuable to study the cleavage pattern of endogenously expressed hepaCAM. However, as we have reported previously, this gene is frequently lost in diverse human tumors (Mon et al., 2005a, b, 2008). Moreover, the cytoplasmic domain of hepaCAM is highly phosphorylated (Moh et al., 2005b), making it difficult to generate antibodies against the cytoplasmic region. Therefore, our research was based on transfection studies with the hepaCAM deficient cell line MCF7 and detection of the V5 tagged hepaCAM by an anti-V5 antibody. Similar transfection studies have been performed in other cell lines such as HeLa, HepG2, HCT116, NIH3T3, etc., and the exogenously expressed hepaCAM constantly shows an additional band at a smaller size than the expected full-length, suggesting that the proteolytic cleavage is a common process for hepaCAM in multiple cell types.

5.2.2 The cleavage product of hepaCAM contains mainly the cytoplasmic domain

By comparing the molecular sizes we concluded that the cleavage product of hepaCAM contains mainly the cytoplasmic domain. For many transmembrane proteins, the extracellular portion is often responsible for ligand binding or receiving other extracellular signals, while the cytoplasmic domain is usually involved in
intracellular signal transduction that leads to functional changes. Although the exact function of the cytoplasmic domain of hepaCAM is not clear, several motifs related to protein-protein interaction or posttranslational modifications have been identified in the cytoplasmic region. The PROSITE and PSORT II programs predicted a proline-rich region (residues 325-385) with 6 SH3-binding domains, a nuclear localization signal (residues 265-271), a prokaryotic membrane lipoprotein lipid attachment site (residues 252-262), a tyrosine sulfation site (274-288), a casein kinase II phosphorylation sites (residues 331-334), and four protein kinase C phosphorylation sites (residues 266-268, 357-359, 371-373, 394-396).

5.2.3 The cleavage product of hepaCAM is membrane tethered

Subcellular fractionation showed that while the full length hepaCAM was found in both cytosol and membrane fractions, the cleavage product of hepaCAM was predominantly detected in membrane fraction, indicating that this fragment is membrane tethered. Since the fractionation kit does not differentiate plasma membrane from membranes of intracellular organelles, it is possible that the cleavage process occurs on both types of membranes. It is well known that the Golgi complex is an important site for proteolytic processing. Most secretory proteins and some plasma-membrane proteins undergo post-Golgi proteolytic cleavages that yield the mature, active proteins. Generally, proteolytic maturation occurs in secretory or transport vesicles carrying proteins from the trans-Golgi network to the cell surface (Xu and Shields, 1993; Sambamurti et al., 2006).

The membrane tethering property also indicates that the cleavage product contains at least part of the transmembrane domain. This is in line with the Western blot result
that the size of the cleavage product was slightly larger than the cytoplasmic domain alone. Therefore the site of cleavage is probably around the transmembrane domain approaching the extracellular side.

5.2.4 PMA has no effect on hepaCAM cleavage

PMA is a phorbol ester and an activator of PKC. The effects of PMA on PKC result from its similarity to one of the natural activators of classic PKC isoforms, diacylglycerol. PMA has been widely documented to stimulate cleavage of cell surface proteins (Hooper et al., 1997; Werb and Yan, 1998). Exposure to PMA up to 2 h only up-regulated the total protein expression of hepaCAM. Prolonged treatment with PMA up to 24 h further enhanced the expression level and resulted in an increase of the ratio between the cleavage product over the full-length of hepaCAM. However, this increase was probably due to the saturation of the full-length protein at later time points. It has been reported that the effects of PMA on proteolytic processing is rapid (Clemens et al., 1992); therefore we concluded that PMA has no effect on hepaCAM cleavage.

PMA is able to increase the expression of many proteins primarily due to the activation of PKC (Kohutek et al., 2009; Jolly-Tornetta and Wolf, 2000). Using a PKC inhibitor GF109203X, we showed that the PMA-induced up-regulation of hepaCAM is indeed through PKC. However, when examining the mRNA level, unlike I-CAM which showed an obvious increase, the expression of hepaCAM was
not changed. Probably PMA affects hepaCAM expression through an unconventional pathway at posttranscription level.

5.2.5 Calcium influx promotes hepaCAM cleavage

Ionomycin enhances Ca\(^{2+}\) influx by stimulating store-regulated cation entry at the plasma membrane (Morgan and Jacob, 1994). We showed that Ca\(^{2+}\) influx induced by ionomycin promoted hepaCAM cleavage. Such effect could be diminished by the Ca\(^{2+}\) chelator EDTA, supporting the role of Ca\(^{2+}\) influx in inducing hepaCAM cleavage. Elevation in intracellular Ca\(^{2+}\) concentration is known to activate PKC (Bootman and Berridge, 1995). However, the PKC inhibitor GF109203X failed to exhibit any inhibitory effect on ionomycin-induced hepaCAM cleavage, implying that the downstream PKC activation is not required for activation of this Ca\(^{2+}\) influx-initiated pathway.

Increased intracellular Ca\(^{2+}\) level is known to transduce diverse intracellular signaling (Berridge et al., 1998). The proteolytic cleavage of other cell surface molecules, including E-cadherin, TGF-\(\alpha\), c-kit receptor and HB-EGF, can also be enhanced by extracellular calcium influx (Ito et al., 1999; Pandiella et al., 1992; Yee et al., 1993; Dethlefsen et al., 1998). These data suggested the existence of a common pathway for the induction of proteolytic cleavage through calcium mobility, though the underlying mechanism remains to be elucidated.
5.2.6 Proteasome is involved in hepaCAM cleavage

Proteasome inhibitors are usually used to stabilize cleavage products that are subjected to proteasome degradation. For example, the residual membrane-tethered cleavage product of E-cadherin could not be detectable by Western blot analysis. However, it becomes apparent by treatment with the proteasome inhibitor MG132 (Ito et al., 1999). Unlike other proteins, treatment of MG132 did not reveal any putative degraded protein of hepaCAM, nor was the cleavage product increased. In contrast, the cleavage was inhibited, suggesting an involvement of proteasome in hepaCAM cleavage. It is of note that the main function of proteasome is to degrade unneeded proteins by non-specific proteolysis (Rivett, 1993). On the other hand, recent studies have shown that proteasome can cleave its substrate in a specific manner. Sorokin et al. (2005) have demonstrated that Y-box-binding protein 1 (YB-1) undergoes a limited proteolysis by the 20S proteasome (Sorokin et al., 2005). Other examples of proteins undergoing limited proteasomal cleavage include the translation initiation factors eIF4G and eIF3a (Baugh and Pilipenko, 2004). The mechanism of hepaCAM cleavage mediated by proteasome remains unknown.

5.2.7 The secretases are not involved in hepaCAM cleavage

Besides proteasome, MG132 exhibits inhibitory effects on other enzymes, including β-, γ-secretases and cysteine proteases (Seinhilb et al., 2001; De Strooper et al., 1999; Tsubuki et al., 1996). Secretases are enzymes that cleave transmembrane proteins. The most well known example is the effects of secretases act on the amyloid precursor protein (APP) that cleaves the protein into three fragments. Sequential cleavage by β-secretase (BACE) and γ-secretase produces the amyloid-β peptide
fragment that aggregates into clumps called "plaques" in the brains of Alzheimer's disease patients. If $\alpha$-secretase acts on APP first instead of BACE, no amyloid-$\beta$ is formed because $\alpha$-secretase recognizes a target protein sequence closer to the cell surface than BACE (Vassar et al., 1999).

It has been reported that MG132 inhibits the cleavage of Notch-1 by $\gamma$-secretase (De Strooper et al., 1999). Brown et al (2000) showed that $\gamma$-secretase is involved in the regulated intramembranous proteolysis (RIP) (Brown et al., 2000). RIP cleaves only type-1 transmembrane proteins (Struhl et al., 2000) and does not depend on sequence recognition (Xia and Wolfe, 2003). We have shown that hepaCAM is a type-1 transmembrane protein (Moh et al., 2005a), offering a clue that hepaCAM may be a substrate of RIP. However, $\gamma$-secretase inhibitor failed to suppress hepaCAM cleavage, ruling out that hepaCAM is an RIP substrate. It has also been shown that MG132 blocks maturation of the APP Swedish mutant by preventing its cleavage by $\beta$-secretase (Steinhilb et al., 2001). Interestingly, the inhibitor of $\beta$-secretase leads to an accumulation of the cleavage product, suggesting that, as a negative regulator, $\beta$-secretase may indirectly be involved in hepaCAM cleavage.

5.2.8 Cysteine proteases are involved in hepaCAM cleavage

Cysteine proteases are a family of enzymes that have a common mechanism involving a nucleophilic cysteine thiol in a catalytic triad. Among members of cysteine protease family, calpains as well as cathepsin B and L can be inhibited by MG132 (Tsubuki et al., 1996). Calpains belong to the family of calcium-dependent, non-lysosomal cysteine proteases expressed ubiquitously in mammals and many other organisms. The calcium-dependent activity, intracellular localization, along with the limited,
specific proteolysis on its substrates, highlight calpain’s role as a regulatory, rather than a digestive protease (Ohno et al., 1994). Two isoforms of calpains, calpain 1 and calpain 2 (or µ-calpain and m-calpain), have been identified. They have different calcium requirements, i.e. micro- and nearly millimolar concentrations of Ca^{2+} within the cell, respectively (Glass et al., 2002). Cathepsin B and L are two the most abundant lysosomal proteases, having concentrations as high as 1 mM (Turk et al., 2000). They are ubiquitous enzymes whose functions have been proposed to extend beyond the confines of the lysosomes. For example, in addition to being localized in the lysosome, cathepsin B (or alternatively spliced variants of cathepsin B) can be found on the surface or in the periplasmic space of tumor cells (Roshy et al., 2003; Sinha et al., 2001; Sameni et al., 1995). Using inhibitors of calpain and cathepsin, we showed the possible involvement of calpain-1 and cathepsin B in hepaCAM cleavage. It is noteworthy that the function of calpain is dependent on calcium and calcium influx is able to increase calpain activity (Huang and Wang, 2001). Thus an elevated calpain activity may partly explain the promoting effect of ionomycin on hepaCAM cleavage.

5.2.9 Multiple pathways are involved in hepaCAM cleavage

Collectively, we have demonstrated several pathways that regulate hepaCAM cleavage. The existence of multiple pathways for the regulation of proteolytic cleavage have been shown in other membrane proteins, including transforming growth factor-α (Pandiella and Masague, 1991), Kit ligand (Huang et al., 1992), c-kit receptor (Yee et al., 1993), and heparin-binding epidermal growth factor (Dethlefsen et al., 1998). Each pathway of hepaCAM cleavage may be responsible for a particular
intracellular or extracellular signal, so that hepaCAM could respond to a variety of stimulations in a complex physiological or pathological environment.

5.2.10 Functional significance of hepaCAM cleavage

An interesting feature of hepaCAM cleavage is that it gives rise to a cleavage product containing mainly the cytoplasmic domain. We have previously demonstrated that hepaCAM exhibits anti-proliferative effects on cancer cell lines and promotes cell-ECM interactions (Moh et al., 2005a, b, 2008). Here we have further shown that truncation of the cytoplasmic domain largely reduced the effects of hepaCAM on cell-ECM adhesion and cell motility. Moreover, the mutant hepaCAM without the cytoplasmic domain has lost the inhibitory effects on cell growth and failed to induce G2/M cell cycle arrest. The importance of the cytoplasmic domain has been emphasized in the functional regulation of transmembrane proteins. Alterations of several CAMs at the cytoplasmic region have led to dysfunction of the proteins. One such CAM is the tumor suppressor in lung cancer 1 (TSLC1), which harbors the 4.1 ezrin/radixin/moesin (FERM) and PSD-95/Dig/ZO-1 (PDZ)-interacting motif in the cytoplasmic domain. Deletion of these motifs abrogates tumor suppressor activity of TSLC1 (Mao et al., 2003). Another example is CD44. Using the truncation mutants lacking part of the cytoplasmic domain, Jiang et al. (2002) have shown that the CD44 cytoplasmic domain is required for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells (Jiang et al., 2002). Our findings on hepaCAM reiterate the importance of the cytoplasmic domain in the functions of transmembrane proteins.
Proteolytic cleavage is often viewed as a regulatory switch that can have activating or inactivating effects on protein functions. For example, some pro-enzymes (such as caspases) need to be cleaved and converted to their active forms (Cohen, 1997); on the other hand, cleavage causes the inactivation of many proteins or removal of receptors from cell surface (Gearing and Newman, 1993). It is noteworthy that the cytoplasmic truncated mutant of hepaCAM has impaired function in cell-ECM interaction and cell growth control, implying that the cleavage of hepaCAM has a negative regulatory effect on its own activity. To further prove this hypothesis, a mutant that is resistant to cleavage would be helpful. This would require the identification of the cleavage site and mutagenesis of the residues that are essential for the cleavage process of hepaCAM.
CHAPTER 6 CONCLUSION

6.1 Conclusion

The present study investigates the properties of the novel gene *hepaCAM*, focusing on the connection between hepaCAM and the integrin-dependent signaling as well as the mechanisms and significance of its proteolytic cleavage.

When re-expressed in MCF7 cells, hepaCAM accelerates the initial cell adhesion and spreading on a fibronectin substrate. Moreover, both the velocity and directionality of cell migration are enhanced by the expression of hepaCAM. Through biochemical studies, hepaCAM is shown to be physically associated with integrin β1, the most highly expressed integrin in MCF7 cells; furthermore, hepaCAM up-regulates the protein expression of integrin β1 as well as its active form in a talin-independent manner. Interestingly, hepaCAM regulates the assembly of the focal contacts, the sites where the cells make connections to the ECM substrate, time dependently. The unique regulation allows the cells to adhere to the ECM substrate quickly and adapts to a migration mode in an efficient way. In addition, hepaCAM alters the activity of Rho family GTPases, which are important mediators in integrin-dependent signaling. While the increased Rho activity is associated with accelerated cell spreading, the decreased Rac activity is well connected to the enhanced directionality of cell migration induced by hepaCAM. At last, the altered signaling mediated by hepaCAM results in an altered organization of the actin cytoskeleton, which supports a migratory phenotype.
hepaCAM undergoes a proteolytic cleavage at around the transmembrane region when re-expressed in MCF7 cells. This cleavage generates a 25-kD cleavage product containing mainly the cytoplasmic domain of hepaCAM. The phorbol ester phorbol 12-myristate 13-acetate (PMA), which is a cleavage inducer of many transmembrane proteins, has no effect on hepaCAM cleavage. However, the cleavage is promoted by the Ca$^{2+}$ ionophore ionomycin and the effect could be diminished by the Ca$^{2+}$ chelator ethylenediaminetetraacetic acid (EDTA). In addition, inhibitors of proteasome and cysteine proteases strongly suppress the cleavage of hepaCAM, indicating the involvement of proteasome, calpain-1 and cathepsin B. Furthermore, functions of hepaCAM are significantly impaired when the cytoplasmic domain is cleaved. The cytoplasmic truncated mutant of hepaCAM fails to promote cell-ECM adhesion and migration, and loses the inhibitory effects on cell growth, suggesting a regulatory role of the cleavage in hepaCAM functions.

In conclusion, the data presented to date shed light on the molecular basis of hepaCAM-mediated cell-ECM interaction and a possible mechanism that hepaCAM employs to regulate its function. Further exploration along these directions may provide insight into the role of hepaCAM in cellular behaviors, cancer progression and the application of hepaCAM in diagnosis and/or treatment of human cancers or other diseases.
6.2 Future work

The work presented in this thesis has laid the foundation for further studies. Along with the interesting findings obtained for hepaCAM, several lines of research may be pursued.

The dual functions of hepaCAM in suppressing cell growth and promoting cell motility seem contradictory as hepaCAM has been identified as a putative tumor suppressor. Profiling the expression of hepaCAM in tumors at different stages may reveal how hepaCAM is differentially regulated during the progression of cancer.

The functional significance of hepaCAM cleavage can be further explored. It will be particularly interesting to identify the site of hepaCAM cleavage and generate a mutant resistant to cleavage by site-directed mutagenesis. Such mutant may help us to examine the functions of hepaCAM when the cleavage mechanism is disabled and to better understand the role of the cleavage in regulating hepaCAM functions.

It will be of great significance to explore the \textit{in vivo} role of hepaCAM in development and physiology. Genetic knock-out along with tissue-specific analysis may give evidence how hepaCAM contributes to certain physiological processes. In addition, proteomics-based technologies may facilitate to identify the protein networks that hepaCAM is involved in and give clues to the signaling pathways that are affected by hepaCAM.
REFERENCES


### APPENDICES

#### Antibiotic solutions

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>100 mg/ml in H₂O</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>30 mg/ml in H₂O</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>10 mg/ml in H₂O</td>
</tr>
</tbody>
</table>

All water-soluble antibiotics were filter-sterilized.

#### Solutions for preparation of competent cells and bacterial cultivation

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB medium</td>
<td>1% Tryptone 0.5g Yeast Extract 1% NaCl</td>
</tr>
<tr>
<td>LB agar</td>
<td>1% Tryptone 0.5g Yeast Extract 1% NaCl 1.5% Agar</td>
</tr>
<tr>
<td>CaCl₂ solution</td>
<td>60 mM CaCl₂ 15% Glycerol 10 mM PIPES [piperazine-(N,N')-bis(2-hydroxypropane-(\text{sulfonic acid})] Adjust pH to 7.0</td>
</tr>
<tr>
<td>0.1 M IPTG</td>
<td>1.2 g IPTG (isopropyl-beta-D-thiogalactopyranoside) in 50 ml of H₂O</td>
</tr>
<tr>
<td>X-gal (2 ml)</td>
<td>100 mg X-Gal (5-bromo-4-chloro-3-indolyl-(\beta)-D-galactoside) in 2 ml of (N,N')-dimethyl-formamide</td>
</tr>
</tbody>
</table>
### Solutions for agarose gel electrophoresis and DNA purification from gel

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× TAE buffer</td>
<td>40 mM Tris</td>
</tr>
<tr>
<td></td>
<td>20 mM Acetic acid</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.8</td>
</tr>
<tr>
<td>6× Loading dye</td>
<td>0.25% (w/v) Xylene Cyanol FF</td>
</tr>
<tr>
<td></td>
<td>15% (w/v) Ficoll 400</td>
</tr>
<tr>
<td></td>
<td>0.25% (w/v) Bromophenol Blue</td>
</tr>
<tr>
<td>3 M sodium acetate</td>
<td>Dissolve 123g of sodium acetate in 500 ml of H₂O</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 5.2 with acetic acid</td>
</tr>
</tbody>
</table>

### Solutions for immunocytochemistry

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1× PBS (1 liter)</td>
<td>8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ • 7H₂O, 0.24 g KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.2</td>
</tr>
<tr>
<td>3.7% Paraformaldehyde (50 ml)</td>
<td>Add 1.85 g of paraformaldehyde into 40 ml of 1X PBS.</td>
</tr>
<tr>
<td></td>
<td>Warm to dissolve. Add 5M NaOH dropwise until solution</td>
</tr>
<tr>
<td></td>
<td>clears. Adjust pH to 7.4 and increase volume to 50 ml.</td>
</tr>
<tr>
<td></td>
<td>Filter-sterilize and aliquot. Store at -20°C</td>
</tr>
</tbody>
</table>

### Solution for cell extraction

<table>
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<tr>
<th>Solution</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA buffer</td>
<td>50 mM Tris pH8.0</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1% Triton X-100</td>
</tr>
<tr>
<td></td>
<td>0.5% NaDOC</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
</tr>
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</table>
## Solutions for Western blot

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</tr>
</thead>
<tbody>
<tr>
<td>2× Laemmli sample buffer</td>
<td>125 mM Tris-HCl pH6.8</td>
</tr>
<tr>
<td></td>
<td>4% SDS</td>
</tr>
<tr>
<td></td>
<td>1.8% β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>10% glycerol</td>
</tr>
<tr>
<td></td>
<td>0.01% bromophenol blue</td>
</tr>
<tr>
<td>5× Laemmli sample buffer</td>
<td>62.5 mM Tris-HCl pH6.8</td>
</tr>
<tr>
<td></td>
<td>2% SDS</td>
</tr>
<tr>
<td></td>
<td>5% β-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>10% Glycerol</td>
</tr>
<tr>
<td></td>
<td>0.05% Bromophenol blue</td>
</tr>
<tr>
<td>10% separating gel (10 ml)</td>
<td>2.72 ml H₂O, 3.75 ml Tris-HCl pH 8.8, 3.33 ml 30% acrylamide/bis solution (29:1; Biorad), 100 μl 10% SDS, 100 μl 10% APS (ammonium persulfate), 4 μl TEMED</td>
</tr>
<tr>
<td>4% stacking gel (4 ml)</td>
<td>2.75 ml H₂O, 0.5 ml Tris-HCl pH 6.8, 0.67 ml 30% acrylamide/bis solution, 40 μl 10% SDS, 40 μl 10% APS, 4 μl TEMED</td>
</tr>
<tr>
<td>1× SDS-PAGE running buffer</td>
<td>25 mM Tris-HCl</td>
</tr>
<tr>
<td></td>
<td>0.2 M Glycine</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 8.3</td>
</tr>
<tr>
<td>Stripping buffer</td>
<td>25 mM Glycine</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 2.0</td>
</tr>
<tr>
<td></td>
<td>1% SDS</td>
</tr>
<tr>
<td>Towbin transfer buffer</td>
<td>25 mM Tris</td>
</tr>
<tr>
<td></td>
<td>192 mM Glycine</td>
</tr>
<tr>
<td></td>
<td>20% v/v Methanol</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 8.3</td>
</tr>
<tr>
<td>10× TBS</td>
<td>0.2M Tris base</td>
</tr>
<tr>
<td></td>
<td>1.37M NaCl</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.6</td>
</tr>
<tr>
<td>1× TBS</td>
<td>20 mM Tris base</td>
</tr>
<tr>
<td></td>
<td>137 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>Adjust pH to 7.6</td>
</tr>
<tr>
<td>1× TBS/T</td>
<td>0.1% Tween-20 in 1× TBS</td>
</tr>
<tr>
<td>Blocking buffer</td>
<td>Dissolve 5% non-fat milk powder (Anlene) in 1× TBS buffer</td>
</tr>
</tbody>
</table>
PUBLICATIONS


Conferences/Presentations

Oral Presentations

Zhang T, Moh MC, Lee LH, and Shen S. Multi-signaling pathway-mediated cleavage of the cytoplasmic domain of hepaCAM, a process that alters the functions of hepaCAM in the human breast carcinoma MCF7 cells. *The 14th World Congress on Advances in Oncology and 12th International Symposium on Molecular Medicine*; 2009 Oct 15-17; Loutraki, Greece. Abstract Number: 121 (Chairperson of one session)

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Zhang T, Moh MC, Lee LH, and Shen S. Calpain is the main element involved in the proteolytic cleavage of hepaCAM, a novel immunoglobulin-like cell adhesion molecule. The 9th International Congress on Cell Biology; 2008 Oct 7-10; Seoul, Korea.