Integrin signaling modes controlling cell migration and metastasis

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Chapter 1

General introduction and scope of this thesis

General introduction

Cell adhesion

The assembly of tissues and organs is dependent on adhesion. In addition to providing structure, it coordinates cues from the surrounding environment to regulate cellular processes such as differentiation and growth during embryonic development and tissue morphogenesis (Hynes, 1987; Hynes and Lander 1992; Hynes 1992). It also regulates pathological processes such as tumor invasion and inflammation, etc. There are two principal types of adhesion: cell-cell and cell-matrix adhesion. Cell adhesion is highly dynamic: adhesion structures contain a large network of proteins whose interactions and conformation is regulated by extracellular cues (Zaidel-Bar et al., 2007). Cell adhesion molecules (CAMs) are specialized integral membrane proteins that mediate cell-cell (homotypic and heterotypic) and cell-matrix adhesion. These adhesions assemble cells into tissues and facilitate communication between cells and their environment. There are four major CAM families: cadherins, immunoglobin (Ig) superfamily members, integrins, and selectins (Cavallaro and Dejana 2011; Hynes 1999; Juliano, 2002).

Distinct classes of ECM adhesion

Distinct classes of extracellular matrix (ECM) adhesion, each consisting of a distinct subset of proteins, exhibit a characteristic subcellular distribution and participate in different signaling events (Yamada and Geiger 1997). Types of cell-matrix adhesion structures include: i) focal complexes (FC), which are small, transient structures, which usually arise immediately behind the leading edge of spreading or migrating cells. These adhesions support nascent filopodial growth and lamellipodia. ii) Focal adhesions (FA) most commonly studied are larger mature structures, which are arise from FCs containing signalling and actin-binding proteins responsible for providing mechanical stability and enabling tractional forces. iii) Fibrillar adhesions (FB) (Geiger et al 2001), which have been considered to originate from a subset of FA are highly stable elongated structures that run parallel to bundles of fibronectin (FN) *in vivo* and are sites of localized matrix deposition and FN fibrillogenesis (Pankov et al.,2000; Zamir et al., 2000). vi) 3D matrix adhesions are fibrillar-ECM adhesion structures that

are dependent in $\alpha5\beta1$ integrin-FN interaction (Yamada,Pankov, and Cukierman, 2003). v) Podosomes and invadopodia are adhesion structures associated with sites of proteolytic degradation of ECM (Linder and Kopp, 2005). Hemidesmosomes (HD) are epithelial specific adhesion structures that link intermediate filaments to ECM (Green and Jones, 1996, Litjens et al., 2006) and are found in epithelial tissues, such as the skin and intestine. There are two type of HDs: Type II is usually found in intestine and comprised of $\alpha6\beta4$ integrin and plectin. Type I, which is established in skin, contains $\alpha6\beta4$ integrin, plectin, tetraspanin CD151, and bullous pemphigoid (BP) antigen 180. Integrin α6β4 and plectin play an essential role in HD formation (Borradori and Sonnenberg 1999; Litjens et al 2006, Spinardi, et al., 1993)

FC, FA, FB, and podosomes may represent a continuum of related adhesions whose structure depends on the protein composition, localization, and proteolytic capabilities. Recent analyses have revealed differences in concentration and post-translational modifications of adhesion proteins among the different adhesion types. For example, transient FC do not contain zyxin but are rich in phosphotyrosine, talin, and $\alpha v\beta3$ integrin (Zaidel-Bar et al., 2004). By contrast, highly stable FB do not contain $\alpha v\beta3$ integrin, phosphorylated (active) focal adhesion kinase (FAK), paxillin and phosphotyrosine, but do have $\alpha5\beta1$ integrin and tensin (Zaidel-Bar et al 2004). FA encompasses FC components and additional proteins like $\alpha5\beta1$ integrin and zyxin and are enriched in phosphotyrosine (Zaidel-Bar et a, 2003; Zamir et al., 2000). Finally, podosomes have an actin core surrounded by tyrosine phosphorylated proteins and several typical FA proteins, such as vinculin and talin and show concentration of proteases (Linder and Kopp, 2005). The functional relevance of these differences in molecular composition is not fully known, but it is likely that distinct populations of proteins will convey distinct mechanical properties to each adhesion.

Integrins are the major mediators of cell-matrix adhesion and also serve as one of the CAM active in cell-cell adhesion. The engagement of integrin to the ECM initiates the adhesion process. Upon interaction with the ECM, integrins are activated by means of a conformational change that permits the receptor to interact with cytoplasmic proteins. Talin is one of the first adaptor protein to bind to the integrin cytoplasmic region Wegener et al., 2007). It's interaction with the β-subunit cytoplasmic tail,

which enhances ligand affinity, which is followed by the clustering of other activating integrins to facilitate strong adhesion formation (Carman and Springer, 2004)

Integrin family

The term integrin was introduced by Tamkun and Hynes (Tamkun et al., 1986) to describe the receptor's function of integrating the ECM network to the actin cytoskeletal network. As members of a membrane glycoprotein superfamily, integrins are transmembrane cell surface receptors consisting of an α- and β-subunit. From 18 α subunits and 8 β subunits, there are 24 heterodimers known to be formed in humans. The assortment of integrins allow for adhesion to probably all ECM proteins, by which cells can promote distinct intracellular signaling responses to changes in ECM composition.

Different types of integrins can be expressed in a cell-type specific manner; thus some integrins such as α5β1, αvβ3 and αvβ6 are associated with migration and proliferation in various cell types whereas other integrins are expressed in selective cell types. Examples of such cell-type specific integrins include αIIβ3 in platelets and α6β4 in epithelial cells (Pierschbacher and Ruoslahti, 1984).
There exists functional redundancy among the integrins in particular processes, such as wound healing. The expression of several integrins (α2β1, α3β1, α9β1) following tissue injury might act as a safe-keeping mechanism ensuring adhesion of the epidermis to any component of the provisional matrix during re-epithelialization (Hunt et al., 1999; Hynes and Zhao 2000; Margadant et al., 2010).

β-subunit

The β-subunit had been extensively studied, whereas the different α-subunits have been less investigated. As reported in previous studies, the β-subunit's cytoplasmic tail is highly conserved and essential for many integrin functions. The removal of the β−subunit cytoplasmic tail inhibits integrin-mediated cell adhesion, cell spreading, cell migration, FAK phosphorylation, β− subunit localization to FA, reduced ligand-binding activity, and activation of signaling proteins (Shattil, 2009; Nieves et al., 2010; Liu, Calderwood, and Ginsberg 2000).

The role of the β-subunit cytoplasmic domains particularly in β1 and β3 integrin has been studied using mutations. There is a number differences between β1 and β3 integrins, for example, Leavesley et al 1993 showed that β1 and β3 integrin-mediated distinct signaling pathway in endothelial cells. They demonstrated that the cellular migration of cells attached to vitronectin through αvβ3 is calcium dependent. In contrast, if the cells attach to collagen through α2β1, migration is calcium independent. Even more strikingly, binding to a single ECM protein, FN through either α5β1 or αvβ3 leads to highly different cytoskeletal organizations and patterns of cell migration (this thesis). It has been reported that in order to regulate cell migration, PKCβ-RACK1 complex must bind to the β3 cytoplasmic tail; whereas β1-mediated migration relies on the direct binding of PKCα and ε. This further shows that both β1 and β3 integrins are connected to similar however distinct signaling pathway (Besson et al., 2002; Ng et al.,1999; Webb et al 2002; Buensuceso et al., 2005).

Interestingly, β1 and β3 integrins coordinate each other function. For instance α5β1 ligation induces calmodulin-dependent kinase II (CAMKII) activation to mediate cell migration, which is inhibited by ligation of αvβ3. Furthermore, it was reported that inhibition of αvβ3-PKD1 interaction upon platelet-derived growth factor stimulation (PDGF) hinders Rab4-dependent recycling of αvβ3 (Blystone et al.,1999; Kim, Harris, and Varner, 2000). This results in an increased recycling of α5β1 through a process that involves association with the Rab-coupling protein (RCP)-Rab11 complex (Woods et al., 2004; White et al.,2007; Caswell et al., 2008).

Integrin structure

The α- and β-subunits have a large extracellular domain, a short transmembrane domain and a short cytoplasmic domain. The extracellular region of β-subunits typically consists of ~750 amino acid residues, and the α-subunit has up to ~1000 residues. Both subunits participate in the ligand-binding head domain. Within this region, the α-subunit contains a divalent cation (ca^{2}+ and Mg^{2}+) binding site and a seven bladed β-propeller domain. A subset of α-subunits also incorporates an I-domain (a.k.a A domain) in their ligand-binding domain, which possesses a conserved metal ion–dependent adhesion site (MIDAS) required for ligand binding. Positioning of the

ligand-binding domain in the α-subunit is mediated by a thigh domain, β-knee, and two calf domains.
The β-subunit ligand-binding domain contains a β-I domain, which is analogous to the α-subunit's I-domain. Positioning of the β ligand binding domain occurs through a hybrid domain, a PSI (plexin/semaphorin/integrin) domain and four epidermal growth factor (EGF) domains.

While cytoplasmic tails of integrins are very short, the β4 cytoplasmic domain forms an exception: it has a very large (1000 a.a) cytoplasmic domain that connects to intermediate filaments rather than the actin cytoskeleton (Litjens et al, 2006 Suzuki and Naitoh, 1990; Hogervorst et al., 1990; Rezniczek et al.,1998).

Signaling

Integrins can transmit signals bidirectionally: integrin-mediated adhesion induces intracellular signaling cascades (outside-in signaling) and intracellular stimuli regulate integrin-mediated adhesion by controlling integrin affinity (inside-out signaling).

Inside-out signaling – controlling integrin affinity

Electron microscopy, structural analysis, and mutation studies have identified integrins in two conformation states: low affinity (inactive form) and high affinity (active form) (shimaoka et al., 2002; Liddington and Ginsberg 2002; Hughes et al., 1996; Calderwood et al., 2004). In the inactive state, the integrin extracellular region is bent and the cytoplasmic tails of α- and β-subunits are close together. The interaction between the α- and β-tails stabilizes the inactive conformation. In its active state, the integrin straightens out and the cytoplasmic tails are separated. The mechanism of integrin activation involves binding of the cytoplasmic protein, talin to the β cytoplasmic tail. The PTB domain within talin's F3 subdomain binds to the β integrin tail, disrupting a salt bridge between the α- and β-tail. As a result, the cytoplasmic tails separate, a conformation change occurs, and the integrin ectodomain is extended (Tadokoro et al., 2003). More recently, it has been shown that in order to achieve maximal integrin activation, assistance of another anchoring protein, kindlin is required. The binding site for kindlin in the β-tail is distinct from the talin binding region (Moser et al., 2008). How the effects at the cytoplasmic site are propagated to the ligand-binding head is

debated and a “deadbolt” model as well as a “switchblade” model has been proposed Takagi and Springer, 2002; Zhu et al., 2007; Liddington,2002; Luo et al.,,2007; Anaout, Mahalingam, and Xiong, 2005).

Bivalent cations critically regulate ligand recognition by the head domain. The major role of cations is to promote a conformation in which the ligand-binding site is exposed (Dransfield et al., 1992; Mould et al., 1995; Bazzoni et al., 1995;Oxvig and Springer 1998,1999). In addition to affinity regulation, integrin clustering is an important factor contributing to adhesion strengthening, whereby post-adhesion accumulation of receptor–ligand bonds contributes to overall adhesiveness (avidity regulation). Clustering of integrins can also occur from integrin association with soluble multivalent ligands. In a ligand-independent manner, valency may also contribute to cellular polarization in which integrins cluster at the leading edge of a migrating cell (Van Kooyk and Figdor, 2000; Stewart and Hogg, 1996, Sampath et al,. 1998). The complexes that form as a result of integrin clustering contain a variety of proteins that facilitate crosstalk between other signaling pathways.

Integrin deactivation is mediated via phosphorylation of tyrosines in the β-tails, which interferes with acidic and hydrophobic interactions between the β-tail and talin, thus causing changes in conformation that reduce ligand-binding affinity. Alternatively, association with negative regulators, such as phosphatidylinositol phosphate kinase type Iγ-90 that competes with the β-tail for talin (Calderwood et al., 2004; Ling et al., 2003).

Outside-in and inside-out signaling - associating transmembrane proteins

Integrins do not transmit signals exclusively, but the interaction/cooperation of other transmembrane cell receptors facilitates signal transduction. Such partnership with other membrane receptors enhances affinity for ligand or intracellular signaling, e.g. during cell migration.

Techniques for detecting, isolating and analyzing complexes of transmembrane proteins, for instance, co-immunoprecipitation and fluorescence resonance energy transfer (FRET) have been used to reveal a diversity of transmembrane proteins ranging from integrin-associated membrane proteases, growth factor receptors, immune

receptors, transporters, and channels interacting with integrins. The regulation of ECM degradation is mediated by the integrin interaction with matrix metalloproteases (MMP). For example, MMP1 binds to the I domain of $\alpha 2$ in $\alpha 2\beta 1$), and the MMP2 carboxy-terminal hemopexin-like (PEX) domain interacts with $\alpha v\beta 3$ (Stricker et al., 2001; Brooks et al., 1998; Boger et al., 2001). Glycan phosphatidylinositol (GPI)-linked proteins, such as uPAR (urokinase-type plasminogen activator receptor) bind to the β-propeller domain of $\alpha M\beta 2$ or $\alpha 3\beta 1$ integrin to mediate cell migration, tumor invasion and host defense (Preissner et al., 2000; Simon et al., 2000). The association of uPAR with $\alpha 3\beta 1$ takes place in caveolae, at least in some cells (Wei et al., 2001).
Integrin-associated protein (IAP; CD47) associates with either $\beta 1$ or $\beta 3$ integrins via its IgSF-like domain to form a functional unit that modulates heterotrimeric G-protein activity (Brown and Frazier, 2001; Wang et al.,1999). Transmembrane-4 superfamily (TM4SF) members, a.k.a. tetraspanins have 4 membrane-spanning domain (2 extracellular loops and intercellular N- and C-termini) and form web-like networks (Berditchevski, 2001). Tetraspanin-integrin interactions have been shown in co-immunoprecipitation studies (CD9, CD53, CD63, CD81, CD82, CD151/PETA-3, and NAG-2). Tetraspanin-integrin complexes vary between cell types and one integrin can associate with one or more tetraspanins. Integrins that are in the tetraspanin web are $\alpha 3\beta 1$, a4$\beta 1$, $\alpha 6\beta 1$, $\alpha 4\beta 7$, and $\alpha IIb\beta 3$. Integrin-tetraspanin complexes have been implicated in the regulation of cell motility, metastasis and growth, integrin recycling, and directing integrin localization on the cell surface (Maecker,Do, and Levy, 1997; Hemler, 1998; Hemler, 2005; Berditchevski, 2001; Boucheix and Rubinstein, 2001; Hemler, Mannion, and Berditchevski, 1996;Y áñez-Mó, M. *et al*.,1998;Tachibana, I. *et al*.,1997).

It was suggested that growth factor receptors may associate with integrins because of their localization in FA and the regulation of proliferation in response to cell adhesion. Indeed, PDGF receptor and insulin receptor β subunit bind to $\alpha v\beta 3$ integrin (Miyamoto, S. *et al*.,1996; Schneller, Vuori, and Ruoslahti, 1997; Bartfield *et al*., 1993; Vuori and Ruoslahti, 1994. It is speculated that integrin interaction functions to cluster growth factor receptors, which promotes efficient signaling or prevent “early dephosphorylation of growth factor receptors (Hellberg et al., 2009; Karlsson et al.,2006; Ivaska and Heino, 2010). Integrins may also bind growth factors themselves:

α9β1 integrin was found to bind to vascular endothelial growth factor (VEGF) to regulate aniogenesis and lymphangiogenesis (Vlahakis et al. 2007;2005).

Outside-in signaling - associating cytoplasmic proteins

Integrins have no enzymatic activity and depend on binding to intracellular proteins to transduce signaling. The intracellular domains are relatively short which restrict the number of proteins can bind at any one time. Approximately ~150 adhesion proteins have been identified that reside in integrin-mediated adhesion complexes (Zaidel-bar et al., 2007). Linker proteins connect integrins with other cytoplasmic proteins, e.g, talin, α-actinin, and filamin. Signalling proteins, including adapters and kinases, such as Src, FAK, and paxillin mediate downstream signaling. Chaperone proteins, for instance Calnexin, which binds to α6β1 integrin, regulates integrin retention in the endoplasmic reticulum (Lenter and Vestweber, 1994).
Upon ligand-binding and integrin clustering, integrins transduce a signal cascade through hierarchical assembly of these associated proteins. Talin is the first cytoskeletal protein to bind the integrin (thereby increasing integrin affinity - see above). Following integrin activation, vinculin interacts with talin and recruits paxillin (Brakebusch and Fassler, 2003). Through phosphorylation by the FAK-Src complex, paxillin becomes activated and recruits other signaling proteins to stimulate further downstream signaling. One example is the activation of the Rho family of small GTPases: by controlling their activity, integrins regulate RhoA-dependent cytoskeletal structures such as stress fibers and FA as well as Rac-dependent structures such as lamellipodia. In this way, integrin signaling controls cytoskeletal dynamics underlying membrane protrusion and cell migration.

Integrin function

Besides providing structure to organs through cell adhesion, integrin-mediated signaling regulates cell behavior such as, proliferation, migration, but also ECM assembly. Integrins mediate binding to - but also formation of ECM networks. In the case of FN, initiation of matrix assembly begins with binding of FN dimers to integrins. Subsequently, in a manner that depends on Rho GTPase-mediated contractility, integrin-bound FN molecules are stretched and this conformational change increases

FN-FN interactions by exposing cryptic FN-binding sites (Mao and Schwarzbauer, 2005). Not all ECM networks depend on integrin interaction: tropocollagens (rod-like collagens) can spontaneously self-assemble to form collagen fibrils during fibrillogenesis (Koide and Nagata, 2005).

Cell migration is crucial for development, wound healing, and tumor metastasis. Integrin traffic (recycling) contributes to the dynamics of adhesion assembly and disassembly, which drive migration. Internalized integrins (disassembly of adhesion structure, FA) from the trailing edge are transported to the newly formed lamellipodium at the leading edge (assembly of adhesion structure, FC) (Caswell and Norman, 2006**;** Pellinen and Ivaska, 2006). In polarized migratory cells, adhesion dynamics at the front differ from those at the rear indicating local differences in integrin affinity or regulation of the adhesion complex (Broussard, Webb, and Kaverina, 2007; Ridley et al., 2003; Schwartz and hortwiz, 2006).

Both cell migration and ECM assembly are important for embryogenesis and tissue repair (wound healing) but also for cancer progression. Synthesis and organization of the ECM has been implicated in formation of a pre-metastatic niche. For example, fibroblasts secrete FN to which bone marrow-derived cells can adhere. Subsequently, the presence of these cells primes the environment for colonization by metastatic tumor cells (Psaila and Lyden, 2009).

Taken together, integrins mediate cell adhesion in a highly controlled fashion. They also participate in the regulation of intracellular signaling cascades. Hence, they are important receptors in many physiological processes. Moreover, they appear to regulate several pathological processes, including cancer progression.

Scope of the thesis

Studies from the mid 60's on malignant cells indicate cell adhesion as a key regulatory factor in many cellular functions (Macpherson and Montagnier, 1964). Altered adhesion-dependency is a key step in malignant transformation. For instance, proliferation and survival (anchorage-dependent processes) are hindered when non-transformed cells are cultured in suspension whereas cancer cells are typically anchorage-independent (Stoker, 1968). Nevertheless, later studies have shown that integrins regulate various aspects of cancer progression (chapter 5 and 7 of this thesis) and might be exploited by the pharmaceutical industry.

The aim of this thesis is to address how integrin-mediated signaling regulates cellular processes that have profound effects on cell morphology, motility, cancer metastasis, and FN fibrillogenesis, and how these findings can be utilized for relevant medical purposes or advancement of drug discovery. The effects on migration and remodeling of FN fibrils are important for cancer progression and embryo development. In **Chapter 2** we discuss how the expression of different FN-binding integrins can have dramatic effects on cell adhesion dynamics and cell motility. In **Chapter 3** we describe how $\alpha 5\beta 1$ and $\alpha v\beta 3$ integrins affect contractility / matrix organization. The ability of the integrin $\alpha 5\beta 1$ hypervariable region of the ligand-binding I-like domain but not that of $\alpha v\beta 3$, with soluble, compact inactive FN molecules appears to affect FA formation, Rho-mediated contractility, and FN fibrillogenesis. Moreover, in chapter 4 we show that the interaction with certain cytoplasmic proteins differes between these two integrins. We report a novel integrin assocating partner MacMarcks (MRP), which regulates cell morphology , actin cytoskeletal organization, and FA distribution through the interaction of $\alpha v\beta 3$ integrin. Interestinly, the interaction of $\alpha v\beta 3$ integrin initiates transcriptional down-regulation of MRP, which leads to cytoskeletal reorganization.

Aberrations in expression level of - or mutations in the integrin, can cause defects in normal cellular function (e.g. anoikis / loss-of-anchorage-induced apoptosis) or affect cancer progression (e.g. enhanced tumor growth). It has been shown that blocking integrins can be a means to prevent progression of cancer or other diseases. However, drug development has reached a bottleneck because of low efficacy and high toxicity. To increase effectiveness of old drugs and improve the speed of drug discovery,

development of proper drug screening approaches is required. In **chapter 5** we explain how integrin-mediated signaling can affect survival, proliferation, differentiation, and disease, and how antagonists of α5β1 and αvβ3 integrins, including disintegrins, RGD peptides, small molecules, and function blocking antibodies, may be of therapeutical value either alone or in combination with existing therapeutical strategies. In **chapter 6**, we describe a novel method that is highly useful for drug screening. Cell-polymer suspensions are microinjected as droplets into collagen gels. Formation time of microinjected derived-cell spheroid (CS) is strongly reduced compared to other methods and can be applied to a broad range of cell types. For high-throughput screening purposes, we have automated this method to produce CS with defined x-y-z spatial coordinates in 96 well plates. We demonstrate the potential of this automated method to develop personalized cancer treatment strategies. Chemical inhibitors are tested on cell lines as well as freshly isolated tumor material from mouse and human biopsies to identify compounds affecting cancer cell invasion/migration. Finally, in **chapter 7**, we show that silencing β1 integrin has a dual effect on cancer growth and progression. In an orthotopic mouse model, growth of β1-deficient breast cancers is significantly reduced in accordance with other studies; however intravasation and lung metastasis are highly increased. We demonstrate that β1 integrin depletion leads to drastic cellular reprogramming, which involves down-regulation of E-cadherin by affecting the ZEB and mir-200 families, causing a switch in migration strategy and enhanced metastasis.

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