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To my beloved family:
Anastasia, Maximilian, and Norman

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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Chapter 2

Integrin switching modulates cell matrix adhesion dynamics.

Truong H and Danen EHJ. *Cell Adhesion and Migration 2009*

Special Focus: Molecular Mechanism of Adhesion Complex Turnover

Integrin switching modulates adhesion dynamics and cell migration

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Key words: integrin, fibronectin, migration, cytoskeleton, dynamics

When cells are stimulated to move, for instance during development, wound healing or angiogenesis, they undergo changes in the turnover of their cell-matrix adhesions. This is often accompanied by alterations in the expression profile of integrins—the extracellular matrix receptors that mediate anchorage within these adhesions. Here, we discuss how a shift in expression between two different types of integrins that bind fibronectin can have dramatic consequences for cell-matrix adhesion dynamics and cell motility.

Cells attach to the extracellular matrix (ECM) that surrounds them in specialized structures termed "cell-matrix adhesions." These come in different flavors including "focal complexes" (small adhesions found in membrane protrusions of spreading and migrating cells), "focal adhesions" (larger adhesions connected by F-actin stress fibers that are derived from focal complexes in response to tension), "fibrillar adhesions" (elongated adhesions associated with fibronectin matrix assembly), and proteolytically active adhesions termed "podosomes" or "invadopodia" found in osteoclasts, macrophages and certain cancer cells. Common to all these structures is the local connection between ECM proteins outside- and the actin cytoskeleton within the cell through integrin transmembrane receptors. The intracellular linkage to filamentous actin is indirect through proteins that concentrate in cell-matrix adhesions such as talin, vinculin, tensin, parvins and others.[1]

Cell migration is essential for embryonic development and a number of processes in the adult, including immune cell homing, wound healing, angiogenesis and cancer metastasis. In moving cells, cell-matrix adhesion turnover is spatiotemporally controlled.[2] New adhesions are made in the front and disassembled in the rear of cells that move along a gradient of motogenic factors or ECM proteins. This balance between formation and breakdown of cell-matrix adhesions is important for optimal cell migration. Several mechanisms regulate the turnover of cell-matrix adhesions. Proteolytic cleavage of talin has been identified as an important step in cell-matrix adhesion disassembly[3] and FAK and Src family kinases are required for cell-matrix adhesion turnover and efficient cell migration.[4,5] Besides regulating phospho-tyrosine-mediated protein-protein interactions within cell-matrix adhesions, the FAK/Src complex mediates signaling downstream of integrins to Rho GTPases, thus controlling cytoskeletal organization.[6,7] The transition from a stationary to a motile state could involve (local) activation of such mechanisms.

Interestingly, conditions of increased cell migration (development, wound healing, angiogenesis, cancer metastasis) are accompanied by shifts in integrin expression with certain integrins being lost and others gained. Most ECM proteins can be recognized by various different integrins. For instance, the ECM protein, fibronectin (Fn) can be recognized by nine different types of integrins and most of these bind to the Arg-Gly-Asp (RGD) motif in the central cell-binding domain. Thus, cell-matrix adhesions formed on Fn contain a mixture of different integrins and shifts in expression from one class of Fn-binding integrins to another will alter the receptor composition of such adhesions. This may provide an alternative means to shift from stationary to motile.

Indeed, we have found that the type of integrins used for binding to Fn strongly affects cell migration. We made use of cells deficient in certain Fn-binding integrins and either restored their expression or compensated for their absence by overexpression of alternative Fn-binding integrins. This allowed us to compare in a single cellular background cell-matrix adhesions containing $\alpha5\beta1$ to those containing $\alpha v\beta3$. Despite the fact that these integrins support similar levels of adhesion to Fn, only $\alpha5\beta1$ was found to promote a contractile, fibroblastic morphology with centripetal orientation of cell-matrix adhesions[8] (Fig. 1). Moreover, RhoA activity is high in the presence of $\alpha5\beta1$ and these cells move in a random fashion with a speed of around 25 mm/h. By contrast, in cells using $\alpha v\beta3$ instead, adhesions distribute across the ventral surface, RhoA activity is low, and these cells move with similar speed but in a highly persistent fashion.[8,9] Finally, photobleaching experiments using GFP-vinculin and GFP-paxillin demonstrated that cell-matrix adhesions containing $\alpha5\beta1$ are highly dynamic whereas adhesions containing $\alpha v\beta3$ are more static.[9]

It has been observed that $\alpha5\beta1$ and $\alpha v\beta3$ use different recycling routes. Interfering with Rab4-mediated recycling of $\alpha v\beta3$ causes increased Rab11-mediated recycling of $\alpha5\beta1$ to the cell surface. In agreement with our findings, the shift to $\alpha5\beta1$ leads to increased Rho-ROCK activity and reduced persistence of migration.[10] One possible explanation for the different types of migration promoted by these two Fn-binding integrins might involve different signaling and/or adaptor proteins interacting with specific amino acids in their cytoplasmic tails. However, this appears not to be the case: $\alpha5\beta1$ in which the cytoplasmic tails of $\alpha5$ or $\beta1$ are replaced by those of αv

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or β3, respectively, behaves identical to wild type α5β1: it promotes a fibroblast-like morphology with centripetal orientation of cell-matrix adhesions and it drives a non-persistent mode of migration.[8,11] Together, these findings point to differences between α5β1 and αvβ3 integrins in the mechanics of their interaction with Fn, which apparently modulates intracellular signaling pathways in control of cell-matrix adhesion dynamics and cell migration.

How might this work? It turns out that although α5β1 and αvβ3 similarly support cell adhesion to immobilized (stretched) Fn, only α5β1 efficiently binds soluble, folded ("inactive") Fn.[11] We have proposed that such interactions with soluble Fn molecules (possibly secreted by the cell itself) may weaken the interaction with the immobilized ligand thereby causing enhanced cell-matrix adhesion dynamics in the presence of α5β1,[11] (Fig. 1). Preferential binding of soluble Fn by α5β1 could be explained by differences in accessibility of the RGD binding pocket between α5β1 (more exposed) and αvβ3 (more hidden) as suggested by others.[12] If this is the case, immobilization ("stretching") of Fn apparently leads to reorientation of the RGD motif in such a way that it is easily accessed by both integrins.

The issue is considerably complicated by the fact that other recognition motifs are present in the Fn central cell-binding domain. In addition to the RGD sequence in the tenth Fn type 3 repeat (IIIFn10), binding of α5β1, but not αvβ3, also depends on the PHSRN "synergy" sequence in IIIFn9.[13-15] The relative contribution of these motifs is controversial and there is structural data pointing either towards a model in which IIIFn9 interacts with α5β1 or towards a model in which IIIFn9 exerts long-range electrostatic steering resulting in a higher affinity interaction without contacting the integrin.[16,17] Cell adhesion studies have suggested that an interaction of α5β1 with the synergy region stabilizes the binding to RGD.[14,18] Such a two-step interaction may facilitate binding to full length, folded Fn for instance by altering the tilt angle between IIIFn9 and IIIFn10 leading to optimal exposure of the RGD loop, perhaps explaining why αvβ3 (which may not interact with the synergy site) poorly binds soluble Fn.

Others have shown that the RGD motif alone is sufficient for mechanical coupling of αvβ3 to Fn whereas the synergy region is required to provide mechanical strength to the α5β1-Fn bond.[19] It appears that the interaction of α5β1 with Fn is particularly dynamic with various conformations of α5β1 interacting with different Fn binding surfaces, including the RGD and synergy sequences as well as other regions in IIIFn9. Thus, besides the above model based on differential binding to soluble Fn molecules, differences in the complexity and dynamics of interactions with immobilized Fn that determine functional binding strength could also underlie the different dynamics of cell-matrix adhesions containing either α5β1 or αvβ3 (Fig. 1).

Precisely how mechanical differences in receptor-ligand interactions result in such remarkably distinct cellular responses is poorly understood. In addition to effects on cell-matrix adhesion dynamics and cytoskeletal organization it is also associated with different activities of Rho GTPases, indicating that mechanical differences between these two integrins must translate into differential activation of intracellular signaling pathways.[8,9,11] Possibly, different adhesion dynamics due to distinct mechanisms of receptor-ligand interaction result in different patterns of F-actin organization, which, in turn, affects the formation of signaling platforms. It is also possible that differences in the extent of integrin clustering have an impact on the conformation of one or more cytoplasmic components of the cell-matrix adhesions containing either α5β1 or αvβ3. This could lead to hiding or exposing binding sites for signaling molecules (e.g., upstream regulators of Rho GTPases) or substrates. Whatever the mechanism involved, altering the integrin composition of cell-matrix adhesions through shifts in integrin expression as observed during development, angiogenesis, wound healing and cancer progression may be a driving force in the enhanced cell migration that characterizes those processes.

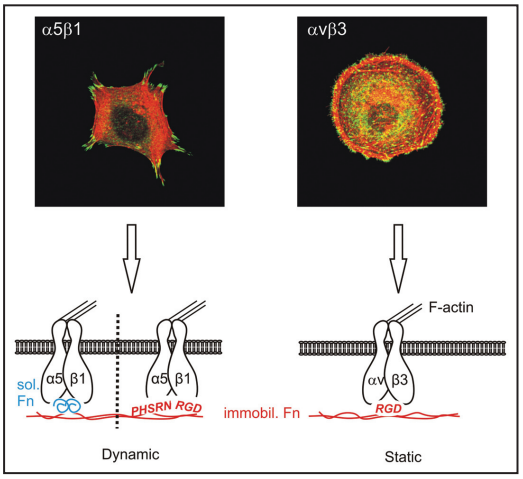

Figure 1. Immunofluorescence images. GE11 cells, epithelial β1 knockout cells derived from mouse embryos chimeric for the integrin β1 subunit endogenously express various αv integrins, including low levels of αvβ3 and αvβ5. Ectopic expression of β1 leads to expression of α5β1 and induced α5β1-mediated adhesion to Fn (left image) whereas ectopic expression of β3 (in the β1null background) leads to strong expression of αvβ3 and induced αvβ3-mediated adhesion to Fn (right image). Adhesions containing either α5β1 or αvβ3 show distinct distribution and dynamics (paxillin; green) and cause different F-actin organization (phalloidin; red). Cartoons: Differences in cell-matrix adhesion dynamics may be explained by differential binding of soluble Fn molecules (blue) or different molecular determinants of the interaction with immobilized Fn (red). See text for details.

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Chapter 3

Regulation of MacMARCKS by integrin β3 expression

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The regulation of MacMARCKS expression by integrin β3

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ABSTRACT

Integrin-mediated adhesion regulates multiple signaling pathways. Our group previously showed that ectopic expression of different integrin β-subunits in the neuroepithelial cell line GE11, has distinct effects on cell morphology, actin cytoskeletal organization, and on focal contact distribution. In this report we have investigated changes in gene transcription levels resulting from overexpression of the integrin β3 subunit. We found that β3 overexpression leads to the transcriptional downregulation of MARCKS related protein (MRP) resulting in a decreased expression of the MRP protein. Furthermore, we show that the Ras/MAPK pathway controls the basal level of MRP expression but β3 overexpression bypasses this pathway downstream of ERK to downregulate MRP. Further studies indicate that a region of the cytoplasmic tail of β3 containing part of the NITY motif is responsible for increased cell spreading and MRP downregulation. However, MRP overexpression failed to inhibit the β3-induced increase in cell spreading while the knock down of MRP expression in GE11 cells did not increase cell spreading. We suggest that the downregulation of MRP by β3 is not required for increased cell spreading but instead that MRP downregulation is a secondary effect of increased cell spreading.

Introduction

Integrin-mediated cell adhesion is essential during development and wound healing and influences the characteristics of malignant tumors [1,2]. Members of the integrin family of hetero-dimeric transmembrane proteins connect the extracellular matrix (ECM) to the actin cytoskeleton and modulate adhesion and migration by means of different downstream signaling pathways [3,4]. In this complex mechanism the regulation of gene expression by integrin-dependent adhesion is also thought to play a role. Studies with monocytes revealed that adhesion to diverse substrata regulates the expression of several genes coding for cytokines and transcription-associated factors (see for review: [5]). Fibroblasts upregulate collagenase, stromelysin, gelatinase and c-*fos* expression when adhered to a matrix of fibronectin and tenascin but not on fibronectin alone [6]. Furthermore, antibodies binding to the integrin subunits α1 and α2 inhibit stromelysin-1 expression [7]. The binding of α5β1 to fibronectin or of αvβ3 to vitronectin increases *Bcl-2* levels through the PI3K–Akt pathway [8]. Also, in *Drosophila*, the presence of the integrin PS1 is required for the normal expression of two genes in the midgut [9]. More recently it was reported that the cell cycle regulator cdc2 is upregulated after re-expression of αvβ3 in β3 knockout cells resulting in increased migration [10]. Moreover, overexpression of β3 in CHO cells leads to a decrease in uPAR expression by bypassing the Ras/MAPK pathway that regulates basal expression of uPAR. The NITY motif in the β3

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cytoplasmic tail is important for uPAR downregulation and binds to the short isoform of the β3 binding protein, β3-endonexin, that has been shown to downregulate *uPAR* transcription [11].

Previously, the profound effects of β1 re-expression in the β1 knockout neuroepithelial cell line, GE11, were investigated [12]. Re-expression of β1 in GE11 cells (GEβ1 cells) results in the loss of cell–cell contacts while the cells acquire a fibroblast-like appearance. On the other hand, overexpression of β3 in this cell line (GEβ3 cells) increased cell spreading and focal contact formation while disrupting cell–cell contacts. Expression of β1 or overexpression of β3 enhanced migration albeit through different modes [12–14]. Since β3 overexpression led to drastic morphological changes we hypothesized that the expression of some genes is regulated by β3. To identify such genes a microarray analysis was performed. Surprisingly, β3 overexpression led to the downregulation of only a single gene that codes for the protein MacMARCKS. MacMARCKS or MARCKS-related protein (MRP) is a small acidic protein with an N-terminal myristoylation site that is inserted into lipid bilayers [15] and a positively charged effector domain (ED) that can bind to negatively charged phospholipids in the plasma membrane [16]. MRP is also thought to bind to actin [17], calmodulin [18] and dynamitin [19], and is phosphorylated at serine residues by PKC [20]. MRP is implicated in the activation of integrins and cell spreading by regulating the cortical actin network [21,22]. The expression of MARCKS, a protein closely related to MRP, is downregulated by the oncogenes v-Jun [23], v-Src [24], c-Ras [25] and H-Ras (dataset from [26]). In contrast, stimulation of BV-2 microglial cells with LPS increases MARCKS and MRP expression via Src kinases [27].

We report that the Ras/MAPK pathway regulates MRP expression in GE11 cells because MEK inhibition led to an increase in MRP expression while RasV12 expression in GE11 cells downregulated MRP expression. Interestingly, β3 overexpression bypassed this pathway downstream of ERK to downregulate MRP expression. Furthermore, we excluded other pathways since we found that overexpression of activated Src, or of β3-endonexin or the inhibition of PI3K did not affect MRP expression. We further show that the presence of the cytoplasmic tail of β3 up to the isoleucine residue of the NITY motif is essential for MRP downregulation but also for increased cell spreading. In another cell line, β3 overexpression also increased cell spreading while MRP expression was downregulated, suggesting that these two effects are linked. However, overexpression of MRP in GEβ3 cells did not inhibit cell spreading while knocking down MRP expression in GE11 cells did not increase spreading. Finally, we present data showing that β3 overexpression regulates the localization of MRP.

Materials and methods

Antibodies and other materials

The following antibodies were used: polyclonal antibodies against MRP (10002-2-Ig, Proteintech Group Inc), MARCKS (m-20, Santa Cruz), monoclonal anti-α-tubulin (clone B-5-1-2, Sigma), monoclonal anti-pan cadherin (clone CH-19, Sigma), monoclonal anti-paxillin (BD Transduction Labs clone 1665), monoclonal ERK2 (BD Transduction Labs clone 33), phospho-ERK rabbit Ab (Cell Signaling #9101). The monoclonal anti-human β3 Ab 23C6 was kindly provided by Dr. Michael Horton (University College London, London, UK). The monoclonal anti-vinculin (clone VIIF9; [34]) and anti-β3 (clone C17) antibodies were kindly provided by Dr. Marina Glukhova (Institut Curie, Paris, France) and Dr. Ellen van der Schoot (Sanquin, Amsterdam, the Netherlands), respectively. Texas Red-conjugated phalloidin was obtained from Molecular Probes. PMA, LY294002 and PD98509 were from Sigma.

Cell lines

GE11 cells have been isolated previously [12] and GE11 cells re-expressing the human β1 or overexpressing the human β3 integrin subunit or expressing the chimera integrin subunits β3-1 or β1-3 were established in our laboratory [12,13]. Cells were cultured in DMEM with 10% fetal calf serum, penicillin and streptomycin. mSCC2 is a mouse squamous cell carcinoma cell line isolated from a skin tumor induced by the two-stage chemical carcinogenesis protocol (Karine Raymond, unpublished results). The human pancreatic adenocarcinoma cell line NP18 has been described previously [28].

cDNA, plasmids and generation of mutants

Full-length MRP was a kind gift from Dr. Deborah Stumpo (National Institute of Environmental Health Science, Research Triangle Park, NC). MRP was cloned into the pEGFP-N1 vector (BD Biosciences, Clontech) by digestion with *Bam*HI and the fragment encoding MRP–GFP was recloned into the retroviral vector LZRS-IRES-zeo [29]. β3 deletion mutant constructs were obtained from Dr. Jari Ylänne (University of Oulu, Oulu, Finland) and cloned into the same retroviral vector. The retroviral expression plasmid encoding H-Ras^{G12V} (Ras^{v12}) was provided by Dr. John Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Both long and short isoforms of β3-endonexin tagged with GFP were obtained from Dr. Meinrad Gawaz (E.H. University of Tubingen, Tubingen, Germany) and cloned into the retroviral LZRS vector. The activated c-Src (Y529F) cDNA was obtained from Upstate Biotechnology. Restriction sites for *Eco*RI and *Not*I were added on the 5′ and 3′ ends of the construct along with a *myc* tag and stop codon at the 3′ end by PCR using the following primers: 5′ GGAATTGAATTCATGGGCAGCAACAAGAGCAA-GAGCAAGCCCAAGGAC and 3′ GGACCTTGCGGCCGCCTAGTT-CAGATCCTCTTCTGAGATGAGTTTTTGTTCTAGGTTCTCCCC-GGGCTGGTACTGTGGGCTC. The fragment was subsequently cloned into the LZRS–IRES–EGFP vector.

Retroviral transductions

Cell lines expressing activated Ras^{V12}, c-Src, MRP–GFP, β3 or deletion mutants of this subunit were established using retroviral transduction. Cells were transduced by adding 1 ml virus-containing supernatant to 10^5 cells in 8 ml medium and incubated for 16 h in the presence of DOTAP (Boehringer). Transduced cells were maintained in fresh medium and sorted three times for a positive GFP signal by FACS® or were

labeled with anti-β3 antibody and FACS sorted after selection with zeocin. A clonal cell line was established from the Src-expressing GE11 cells and used for transduction of β3. Resulting cells were sorted three times for β3 expression.

Microarray analysis

Microarray slides were prepared at the central microarray facility (CMF) at the Netherlands Cancer Institute. A list of genes is available at http://microarrays.nki.nl/download/geneid.html. Cell lines were grown on plastic in normal growth medium and total RNA was isolated, labeled and hybridized as described at http://www.nki.nl/nkidep/pa/microarray/protocols.htm.

Northern blot analysis

Corresponding clones of the identified genes were obtained from the central microarray facility. DNA was isolated, sequenced and used as templates for PCR reactions to obtain a suitable probe for Northern blot analysis. The following primers were used for MRP (AAGGAGACCCCCAAGAAGAA and CTCATTCTGCTCAGCACTGG). Total RNA was isolated using guanidine–isothiocyanate (GIT). Briefly, cells were lysed in a buffer containing 4 M GIT, 25 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.5% sarkosyl. RNA was isolated after phenol chloroform extraction and was precipitated with isopropanol. Northern blots were performed using standard protocols.

Cell labeling and immunoprecipitation

Cells were surface-labeled with ^{125}I using lactoperoxidase as described previously [30]. Cells were lysed in 1% Nonidet P40 in 25 mM Tris–HCl, pH 7.5, 4 mM EDTA, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10 μl/ml leupeptin and 10 μl/ml soybean trypsin inhibitor. Cell lysates were clarified by centrifugation and immunoprecipitations were performed with antibodies bound to protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) or to protein A-Sepharose conjugated with rabbit anti-rat IgG or anti-mouse IgG. Immunoprecipitates were analyzed by SDS–PAGE under non-reducing conditions and visualized using Kodak Biomax XAR film.

Western blot analysis

Cell culture plates containing attached cells were washed with PBS and lysis buffer [50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EGTA, 100 mM NaF, and inhibitor cocktail (Sigma)] was added. Cells were scraped and centrifuged for 2 min at 14,000 rpm. 20 μl of lysate was added to 5 μl 3× SDS sample buffer (Biolabs) and boiled. Samples were loaded onto SDS–PAGE gels, separated and transferred to polyvinylidene difluoride membranes (Millipore) and analyzed by Western blotting followed by ECL using the Super signal system (Pierce Chemical Co.).

Immunofluorescence and flow cytometry

For immunofluorescence cells were fixed in 2% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 5 min with PBS washing in-between steps. Coverslips were subsequently blocked with 2% BSA in PBS for 1 h at room temperature (RT). Coverslips were incubated with primary antibodies for 1 h at RT, washed three times in PBS and incubated with FITC or Texas Red secondary antibody (Jackson ImmunoResearch Laboratories) for 1 h at RT. Slides were mounted in MOWIOL 4–88 solution supplemented with DABCO (Calbiochem) and examined with a confocal Leica TCS NT microscope. For all immunofluorescence experiments, 4 random fields each of three independent experiments were examined of which a representative image was used for publication. For cell sorting cells were trypsinized, washed twice in PBS supplemented with 2% serum and incubated with primary antibody for 1 h at 4 °C. After three washes, cells were incubated with FITC- or PE-conjugated secondary antibody for 1 h at 4 °C. Finally cells were washed and resuspended in PBS with 2% serum and sorted using a FACStar plus® (Becton Dickinson). Cells expressing GFP constructs were trypsinized and washed before being sorted.

RNAi against MRP

A SMARTpool® from Dharmacon consisting of 4 siRNAs (catalog number M-042960-00-0010) against MRP (NM_010807) was used to transfect GE11 cells along with the standard si*CONTROL*® siRNA from Dharmacon as negative control. Cells were transfected using the standard transfection protocol provided by Dharmacon using the transfection reagent DharmaFECT® 1. Protein expression was assessed 48 h after transfection while at the same time cells were fixed and stained for confocal analysis.

Results

Overexpression of β3 in GE11 cells regulates MRP expression

GE11 cells have an epithelial morphology adhering to one another with well-defined cell–cell contacts. They have small, peripheral focal contacts and a thick cortical actin ring present along the circumference of the entire epithelial island. Overexpression of β3 in GE11 cells (GEβ3 cells), causes a reorganization of the actin cytoskeleton resulting in the loss of the cortical actin ring and the formation of short, thick stress fibers connected to a large number of focal contacts and the loss of cell–cell contacts (Fig. 1A).

Since β3 overexpression induced these dramatic morphological changes in GE11 cells, we hypothesized that the regulation of some genes would be changed. To identify these genes we performed a microarray analysis of the GEβ3 cell line using GE11 cells as reference. Genes, whose transcription level differed three-fold or more from that in the reference GE11 cells were selected. Surprisingly, despite the dramatic morphological changes induced by β3 overexpression in GE11 cells, the transcription of only a single gene appeared to be affected. The transcription of this gene, *Mlp*, coding for the protein MRP was downregulated by 72% in GEβ3 cells as compared with GE11 cells. This was confirmed by Northern blot analysis because mRNA levels for MRP were strongly decreased in GEβ3 cells in comparison to GE11 cells (Fig. 1B). To

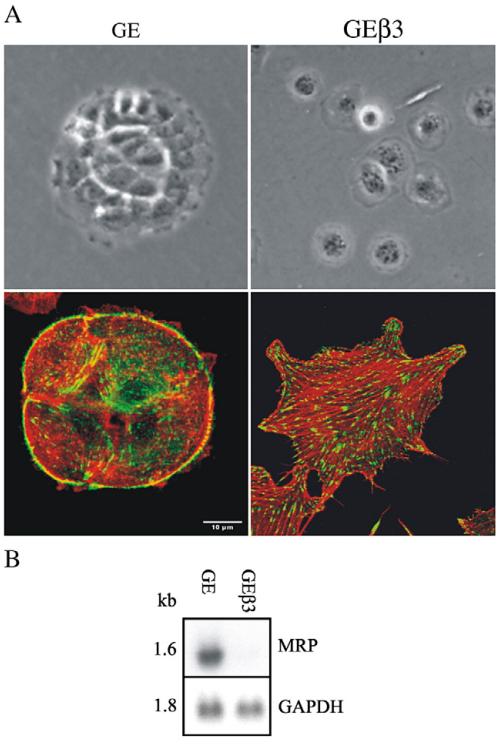

Fig. 1 – Analysis of gene transcription after β3 overexpression. (A) Phase contrast and confocal analysis of GE11 and GEβ3 cells. Cells were stained with anti-paxillin antibodies in green and with phalloidin in red. (B) Total RNA was isolated from GE11 and GEβ3 cells and analyzed using Northern blot. mRNA bands were visualized using radioactively labeled probes.

determine if the decrease in *Mlp* transcription also led to a decrease in MRP expression, lysates of GE11, GEβ3 and GEβ1 cells were analyzed by Western blot. Indeed, MRP expression was diminished after β3 overexpression but remained unchanged in GEβ1 cells (Fig. 2A). To exclude the possibility that the observed downregulation of MRP expression is attributable to clonal variation, we analyzed several independent, bulk-sorted GEβ3 cell populations. MRP expression was diminished in all of them (data not shown). Western blot analysis showed that β3 overexpression had no effect on the expression of MARCKS (Fig. 2A). Therefore, overexpression of β3 in GE11 cells results in the specific downregulation of MRP expression.

The cytoplasmic tail of β3 downregulates MRP expression only when it is associated with the αv subunit

Integrins convert signals from the extracellular environment into intracellular signals via their cytoplasmic tails [3]. We hypothesize that the cytoplasmic tail of β3 is involved in the downregulation of MRP expression. To test this, a chimera of the β3 extracellular and transmembrane domain, fused to the β1 cytoplasmic tail (β3-1) and a construct of the β1 extracellular and transmembrane domains fused to the β3 cytoplasmic tail (β1-3) were expressed in GE11 cells and MRP expression was measured (Fig. 2B). Expression of the chimeras did not affect MRP expression indicating that the presence of the cytoplasmic tail of β3 without the extracellular domain, or vice versa, was insufficient to downregulate MRP expression.

Possibly, the effect on MRP expression depends on the α subunit that is associated with β3. For that reason we identified the integrin complexes present on the surface of the cells expressing full-length β3 or either of the chimeras (Fig. 2C). Both full-length β3 and the β3-1 chimera were associated with the αv subunit while the β1-3 chimera was associated with α5 and/or α3. Therefore, the cytoplasmic tail or the extracellular domain of β3 by themselves cannot affect MRP expression but instead it is the association of the αv and the β3 subunits that mediates this effect.

A distinct region of the β3 cytoplasmic tail is responsible for increased cell spreading and MRP downregulation

To characterize the underlying mechanism responsible for the regulation of MRP by β3, we sought to identify the region within the β3 cytoplasmic tail that mediates the downregulation of MRP expression. MRP expression was measured

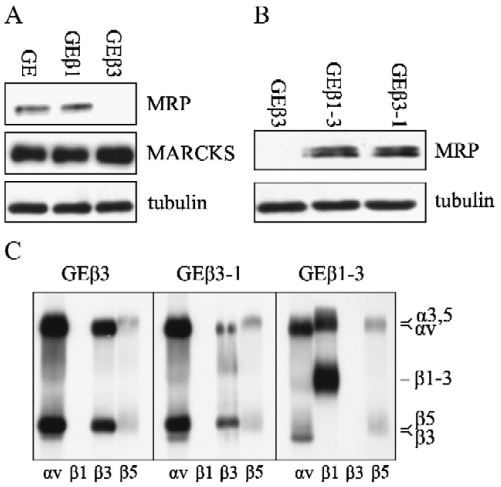

Fig. 2 – $\alpha_v\beta_3$ only downregulates MRP expression. (A) Western blot stained for MRP and MARCKS expression in GE11, GEβ1 and GEβ3 cell lines. Tubulin was visualized on the same blot as the loading control. (B) Western blot for MRP expression on lysates of GEβ3, GEβ3-1 and GEβ1-3 cells with tubulin staining used as loading control. (C) Surface-expressed integrins were labeled using ^{125}I and immunoprecipitated using antibodies against the indicated integrin subunit.

in several cell lines expressing different truncated β3 constructs (Fig. 3A). Our data shows that expression of a construct with a deletion of the five C-terminal residues of β3 representing the complete Src-binding motif (RGT) [31] and the tyrosine and threonine residues of the NITY motif still led to downregulation of MRP expression. However, the expression of further truncated constructs of the cytoplasmic tail of β3 no longer had an effect on MRP expression (Fig. 3B). Interestingly, β3-induced increased cell spreading appeared to depend on the same region of the cytoplasmic tail that influences MRP expression (Fig. 3C). Thus, it is possible that proteins that bind to β3 in the vicinity of the NITY motif are involved in the regulation of MRP expression as well as in increased cell spreading.

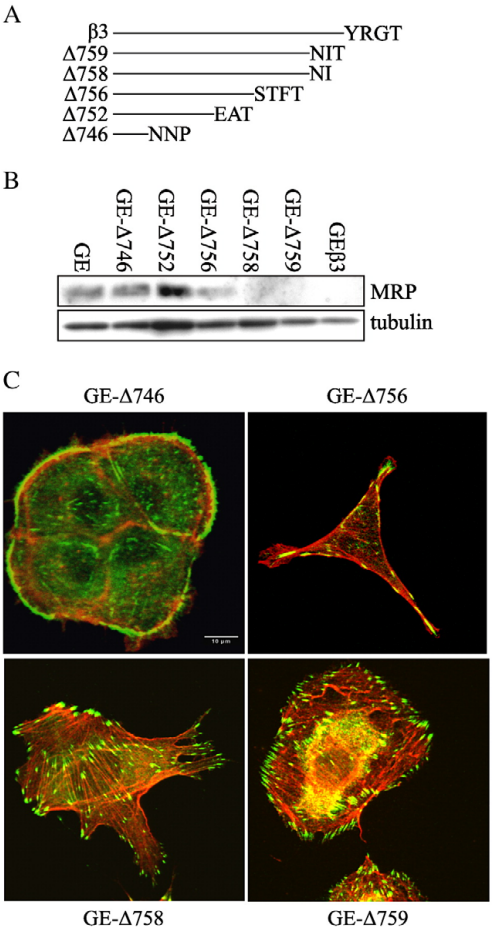

Fig. 3 – The distal NITY motif is essential for downregulation of MRP expression and increased cell spreading. (A) Diagram depicting the deletion mutants of the β3 and showing the last amino acids of each mutant. (B) Cells expressing deletion mutants were analyzed for MRP expression by Western blot. (C) Confocal images of these cells stained for paxillin and F-actin indicate the differences in FC formation, F-actin organization and cell shape.

The Ras/MAPK pathway regulates the basal level of MRP expression in GE11 cells but β3 overexpression bypasses this pathway to downregulate MRP

Stimulation of BV-2 microglial cells with LPS upregulates MRP expression through a Src dependent signaling pathway [27]. Moreover, MARCKS expression is downregulated by v-Src and c-Ras expression in 3T3 cells [25]. Thus, the question arose whether β3 overexpression in GE11 cells downregulates MRP expression through a Src-dependent pathway or the Ras/MAPK pathway. GE11 cell lines were established expressing activated c-Src or expressing activated c-Src together with β3. Western blot analysis of these cell lines indicated that the expression of activated c-Src did not affect MRP expression in the GE11 or β3 overexpressing cell lines (Fig. 4A). Src kinases and protein tyrosine kinases (PTK) were inhibited in GEβ3 cells using PP2 and Herbimycin A, respectively (Fig. 4B). Neither Src nor PTK inhibition restored MRP expression. Therefore, the regulation of MRP by β3 is independent of Src activity.

To test whether the Ras/MAPK pathway is involved in the regulation of MRP expression, GE11 and GEβ3 cells were incubated for increasing periods of time with 25 μM PD98509. ERK phosphorylation levels decreased after an incubation of 1 h. Interestingly, MRP levels increased sharply after 1 h in GE11 cells but not in GEβ3 cells (Fig. 5A). Both cell lines were also incubated for 2 h with increasing concentrations of PD98509. ERK phosphorylation levels were decreased in both cell lines after incubation with 10 μM, but MRP expression only increased in GE11 cells after incubation with 10 μM PD98509 while there was no change in MRP expression in GEβ3 cells (Fig. 5B). To confirm that the Ras/MAPK pathway regulates MRP expression, a Ras^{V12} construct was stably expressed in GE11 cells. Expression of Ras^{V12} resulted in increased ERK phosphorylation along with a decrease in MRP expression (Fig. 5C). Therefore, the Ras/MAPK pathway regulates MRP expression in GE11 cells, but β3 overexpression bypasses the effect of ERK phosphorylation to downregulate MRP expression.

Two other pathways that were previously implicated in gene regulation downstream of integrins were investigated for their possible role in the β3-dependent downregulation of MRP expression. Firstly, it was reported that the short isoform of the β3 integrin-binding protein, β3-endonexin, is important for the downregulation of uPAR expression after β3 overexpression in CHO cells [11]. To investigate if β3-dependent MRP downregulation is mediated by β3-endonexin, constructs with the long or short isoform fused to GFP were expressed in GE11 cells followed by Western blot analysis of MRP (Fig. 4C). MRP expression was not influenced by the expression of either isoform of endonexin. Therefore, β3-induced downregulation of MRP is not dependent on the presence of β3-endonexin. Secondly, it was shown that ligand binding by αvβ3 and α5β1 leads to increased Bcl-2 expression through the activity of the PI3K pathway [8]. We investigated if PI3K plays a role in the regulation of MRP expression by incubating GE11 and GEβ3 cells overnight with the specific inhibitor LY294002 and analyzing MRP expression. The data shows that LY294002 did not alter MRP expression at the concentrations tested (Fig.

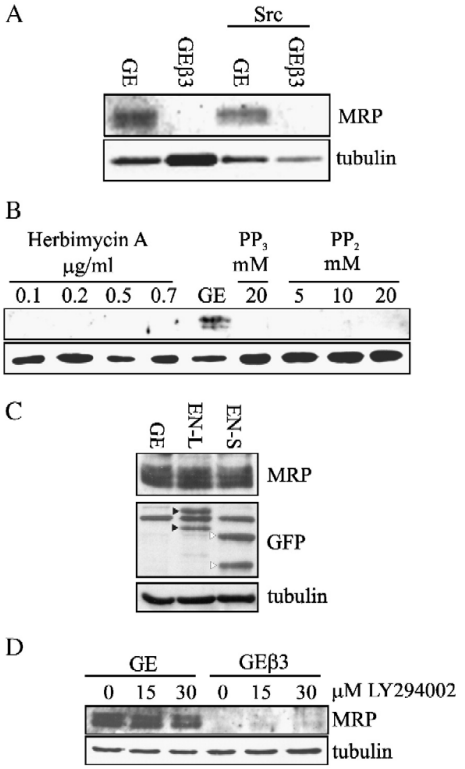

Fig. 4 – MRP expression is regulated independently of Src activity. (A) GE11 and GE11-Src cell lines with or without β3 overexpression were analyzed for MRP expression. (B) GEβ3 cells were incubated with Herbimycin A, PP3 or PP2 and analyzed for MRP expression together with GE11 cells as positive control. (C) Long (EN-L) and short (EN-S) forms of endonexin fused to GFP were stably expressed in the GE11 cell line. Western blot analysis was performed for endogenous MRP levels and the same blot was stained for GFP and tubulin. Note that endonexin is present as two distinct bands indicated with filled arrowheads for EN-L and empty arrowheads for EN-S. (D) GE11 and GEβ3 cells were incubated with 0, 15 or 30 μM LY294002 overnight before being lysed and analyzed for MRP expression by Western blot.

4D). Therefore, the PI3K pathway is not involved in β3-mediated regulation of MRP expression.

MRP downregulation and cell spreading are regulated by β3 in the NP18 cell line

To determine if the downregulation of MRP expression by overexpressed β3 only occurs in GE11 cells, we measured MRP expression in two other cell lines overexpressing β3. In the pancreatic carcinoma cell line, NP18, MRP was down-regulated after β3 overexpression but MRP expression was not affected in the mouse squamous cell carcinoma cell line mSCC2 (Fig. 6A). Paxillin and actin staining of the parental NP18 cells indicated that focal contacts were present in clusters evenly spaced at the cell periphery in close proximity to a cortical actin network. In contrast, in the NP18-β3 cell line the focal contacts present around the periphery of cells were more often connected to thick actin stress fibers extending across the entire cell. However, overexpression of β3 in the mSCC2 cell line did not noticeably affect the morphology of the cells (Fig. 6B). Thus, the effect of β3 overexpression on MRP expression is not limited to the GE11 cell line. Moreover, the overexpression of β3 induced morphological changes in the NP18 cells similar to those seen in GE11 cells.

MRP is not essential for β3-induced effects on morphology but αvβ3 expression does regulate MRP localization

We showed that the downregulation of MRP expression by β3 overexpression is accompanied by morphological changes. To determine if the loss of MRP is responsible for these morphological changes, we silenced MRP expression in GE11 cells using a siRNA SMARTpool® from Dharmacon. The SMARTpool® along with a negative control were transfected

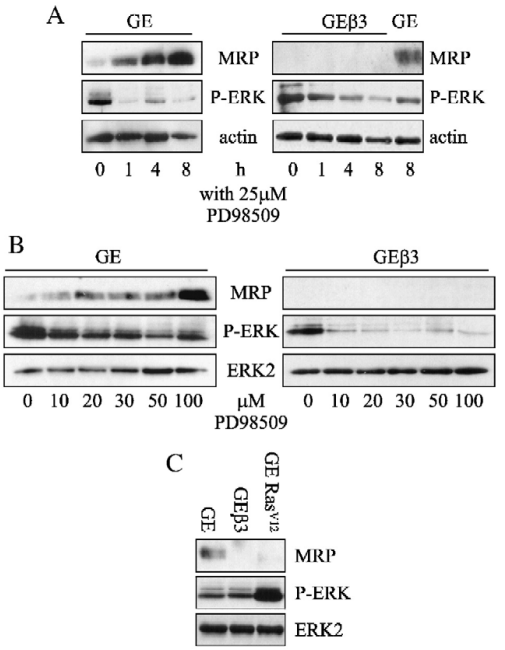

Fig. 5 – MRP expression is regulated by the Ras/MAPK pathway. GE11 and GEβ3 cells were incubated with PD98509 for different periods (hours) (A) or at different concentrations (bottom in μM) (B). MRP and phospho-ERK levels were analyzed with actin or ERK2 as loading control. (C) GE11, GEβ3, and GE Ras^{V12} were analyzed for MRP and phospho-ERK levels with ERK2 as loading control.

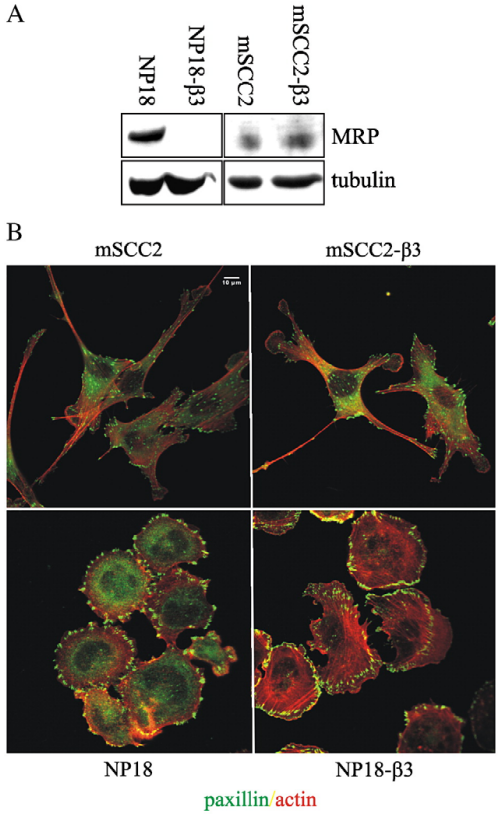

Fig. 6 – Effect of β3 overexpression in NP18 and mSCC2 cells. (A) Western blot analysis for MRP expression of two cell lines, NP18 and mSCC2, overexpressing β3. (B) mSCC2 cells and NP18 cells with or without overexpressed β3 were stained for paxillin and F-actin.

into GE11 cells and MRP expression was assessed after 48 h. Western blot analysis showed that MRP was absent in the SMARTpool® transfected cells while cells transfected with the control siRNA still expressed MRP (Fig. 7A). Cells were also fixed and stained for vinculin, cadherin and F-actin. Cells transfected with the negative control and with the MRP-directed siRNA formed islands with a normal morphology and normal F-actin and vinculin distribution (Fig. 7B). There was no evidence that cell spreading or cell–cell contact formation was affected by the knockdown of MRP. Additionally, MRP was re-expressed in GEβ3 cells using a MRP–GFP cDNA construct. Confocal analysis revealed that MRP–GFP expression had no effect on the morphology of GEβ3 cells (Fig. 8A). The cells remained well spread and the formation of actin stress fibers and focal contacts was not perturbed. To determine if the downregulation of MRP expression in GEβ3 cells is important for the initial spreading of these cells, MRP–GFP was stably expressed in GE11 cells before β3 was overexpressed through retroviral transduction. 24 h after infection, the morphology of cells expressing MRP–GFP and β3 was similar to that of GEβ3 cells (Fig. 8B). Therefore, we conclude that cell spreading is not influenced by the levels at which MRP is expressed. Interestingly, we noticed that the localization of MRP–GFP was altered after β3 overexpression. In line with a previous report showing that MRP is localized at the basolateral membranes of MDCK cells [32], confocal analysis of GE11 cells indicated that overexpressed MRP–GFP was concentrated at the basolateral membrane and partially co-localized with cadherin but not with paxillin (Fig. 8A). In contrast, after β3 overexpression MRP–GFP was diffusely distributed along the entire cell membrane with occasional staining of internal membranes as well as weak actin filament decoration. In conclusion, MRP is not involved in the β3-induced morphological effects, but β3 overexpression clearly influences MRP–GFP localization.

Discussion

It has been suggested that the regulation of gene expression forms part of the mechanism by which integrins control cell migration and invasion [10]. Previously, it was shown that the re-expression of β3 in knockout cells increases cdc2 protein levels [10] and that overexpression of β3 in CHO cells leads to a decrease in uPAR protein levels [11]. In both cell lines migration and spreading were enhanced. Previous work in our laboratory has shown that overexpression of β3 or re-expression of β1 subunit has distinct effects on cell morphology, migration, Rho activation and cofilin phosphorylation [13,14].

We hypothesized that the effect on cell morphology induced by β3 overexpression coincides with changes in the transcription of several genes, possibly including genes such as *uPAR* and *cdc2*. To test our hypothesis we performed a microarray analysis of GEβ3 cells and compared the gene expression profile of these cells to that of the parental GE11 cells. Surprisingly, β3 overexpression resulted in the down-regulation of only a single gene, i.e. the gene coding for MRP. Both uPAR and cdc2 cDNAs were present on the array used for this study but the transcription of these genes was not changed by the overexpression of β3.

Various signaling pathways have been implicated in gene regulation downstream of integrins. One of these pathways was elucidated in CHO cells in which β3 overexpression inhibits *uPAR* transcription. It was found that overexpression of the short isoform of the β3 integrin binding protein, β3-endonexin, also resulted in decreased *uPAR* transcription [11] suggesting that β3-endonexin is the downstream effector of β3 in a pathway that leads to uPAR down-regulation [11]. It was also shown that ligand binding by αvβ3 and α5β1 leads to an increase in Bcl-2 expression that was dependent on the PI3K–Akt pathway [8]. We investigated if these signaling pathways were also involved in the β3-mediated downregulation of MRP expression. We show that overexpression of β3-endonexin in GE11 cells had no effect on MRP expression and we conclude that this pathway does not regulate the expression of MRP. Our data also indicates that MRP expression was not changed by inhibition of the PI3K pathway.

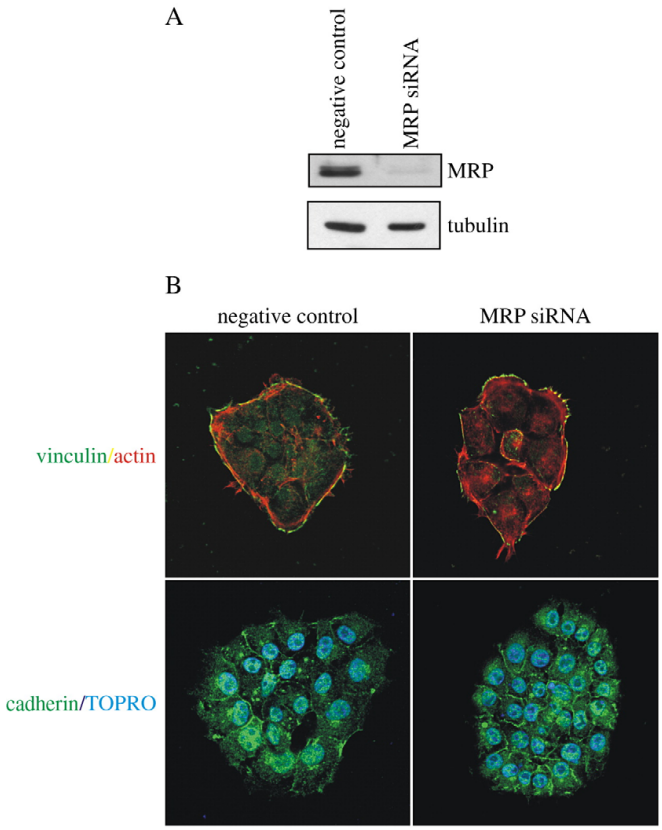

Fig. 7 – RNAi-mediated downregulation of endogenous MRP in GE11 cells. (A) Western blot analysis of MRP expression in GE11 cells transfected with siRNA against MRP and a negative control. (B) GE11 cells transfected with siRNA directed against MRP or with a negative control were fixed and stained for vinculin and actin or for cadherin together with TOPRO that was used as nuclear staining.

On the other hand, a number of soluble factors and signaling intermediates have been identified that affect MRP expression. It was shown that LPS stimulation increases MRP expression in BV-2 microglial cells through the activity of Src kinase [27]. Moreover, transformation of 3T3 cells with v-Src or Ras leads to decreased expression of MARCKS [25]. Our results indicate that active Src does not decrease MRP expression. Intriguingly, the inhibition of ERK phosphorylation caused an increase in MRP expression in GE11 cells but had no effect on MRP expression in GEβ3 cells while Ras^{V12} expression in GE11 cells resulted in increased phospho-ERK levels and decreased MRP protein levels. Therefore, the Ras/MAPK pathway controls the basal level of MRP expression in GE11 cells but β3 overexpression bypasses this pathway downstream of ERK to downregulate MRP expression.

Our studies with β3 chimeras show that downregulation of MRP expression by β3 only occurs when it is associated with the αv subunit. Furthermore, we show that the β3 cytoplasmic tail is essential for MRP downregulation. Within the cytoplasmic tail of β3 several motifs have been identified that are important for different downstream signaling events. For example, the membrane proximal NPxY motif is important for binding to talin and critical for integrin activation, while the distal NITY motif is important for cell spreading although it appears that the tyrosine residue of this motif is not essential [33]. We tested several deletion mutants of β3 and show that the loss of the complete NITY motif does not lower the expression of MRP. Inclusion of the asparagine and isoleucine residues of the NITY motif leads to the downregulation of MRP expression while also causing cells to spread like GEβ3 cells. Therefore, the same region of β3 responsible for MRP downregulation also increases cell spreading. In NP18 cells, β3 overexpression also caused changes in the cytoskeleton while MRP expression was downregulated. On the other hand, overexpression of β3 in mSCC2 cells did not lead to changes in morphology or to the downregulation of MRP expression, emphasizing that β3 overexpression is responsible for both these phenomena. MRP has been implicated in cell spreading and cytoskeletal organization [20,21], both of which are altered in GEβ3 cells,

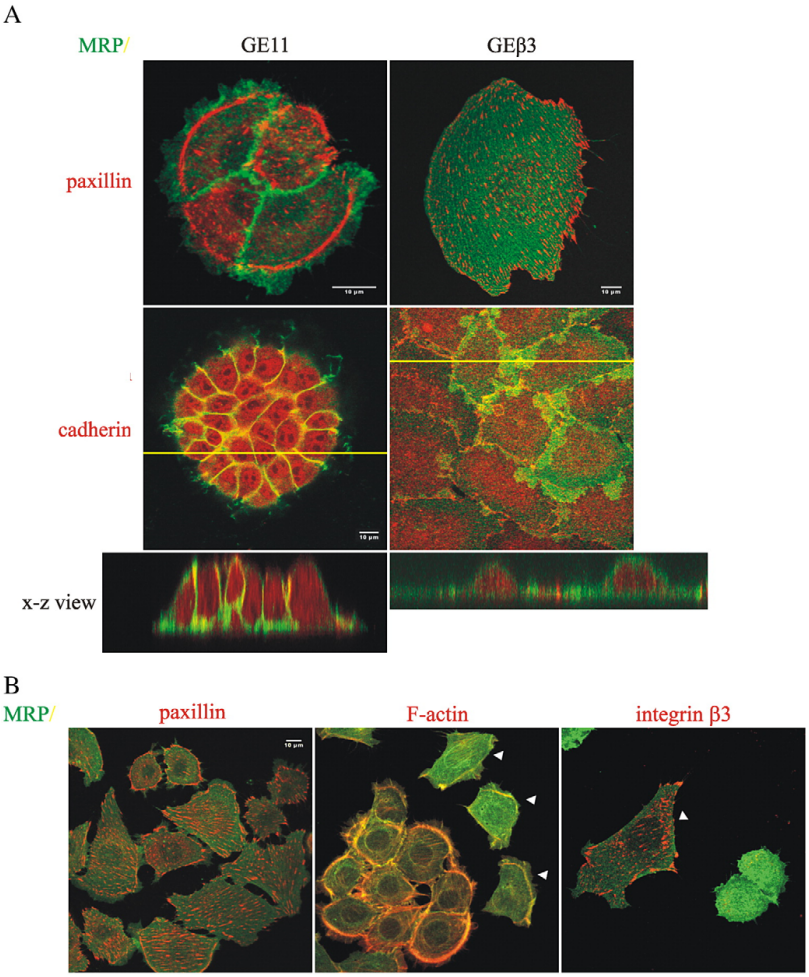

Fig. 8 – Overexpression of MRP has no effect on β3-induced cell morphology. (A) GE11 and GEβ3 cells expressing MRP–GFP were grown on glass coverslips, fixed and stained for paxillin and actin or for cadherin while a *z*-stack was made of a GE–MRP island and of a GEβ3–MRP confluent cell layer stained for cadherin. The vertical slice presented in the right panel is obtained from the region indicated by the white line in the left panel. (B) GE11–MRP cells were grown on slides and infected with β3-retrovirus. After 24 h of infection, cells were fixed and stained for paxillin, F-actin and integrin β3. All the cells in the left panel that stained positively for paxillin have the typical β3-induced morphology while arrowheads in the right panel indicate infected cells.

suggesting that MRP may be important for the β3-induced effects on cell morphology. However, our data indicates that altering MRP expression by knock down in GE11 cells or by overexpression in GEβ3 cells does not influence cellular morphology. We, therefore, suggest that either MRP is not involved in cell spreading or morphology or that it is not the only protein involved and that the stronger effects of other proteins mask the effect of MRP.

In conclusion, our studies show that the expression level of β3 can influence cell morphology and the transcription levels of MRP and that the Ras/MAPK pathway is important for the regulation of MRP expression.

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Chapter 4

Binding of soluble fibronectin to integrin alpha5beta-linked to focal adhesion redistrubtion and contracticle shape

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Binding of soluble fibronectin to integrin α5β1 – link to focal adhesion redistribution and contractile shape

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Summary

Focal adhesions are randomly distributed across the ventral surface or along the edge of epithelial cells. In fibroblasts they orient centripetally and concentrate at a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology. Extensive remodeling of adhesions in fibroblasts also takes part in fibronectin fibrillogenesis, a process that depends on Rho-mediated contractility and results in the formation of a fibronectin matrix. Our current study shows that all these fibroblast characteristics are controlled by the ability of integrin α5β1 to bind soluble fibronectin molecules in their compact inactive conformation. The hypervariable region of the ligand-binding I-like domain of integrin α5β1 supports binding of soluble fibronectin. This supports the distribution of centripetally orientated focal adhesions in distinct peripheral sites, Rho activation and fibronectin fibrillogenesis through a mechanism that does not depend on Syndecan-4. Integrin αvβ3, even when locked in high affinity conformations for the RGD recognition motif shows no appreciable binding of soluble fibronectin and, consequently, fails to support the typical fibroblast focal adhesion distribution, Rho activity and fibronectin fibrillogenesis in the absence of integrin α5β1. The ability of α5β1 integrin to interact with soluble fibronectin may thus drive the cell-matrix adhesion and cytoskeletal organization required for a contractile, fibroblast-like morphology, perhaps explaining why α5β1 integrin, similarly to fibronectin, is essential for development.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/15/2452/DC1

Key words: Adhesion, Cytoskeleton, Extracellular matrix, Rho, Matrix assembly

Introduction

Integrins consist of non-covalently linked α- and β-subunits. Ligands, including extracellular matrix (ECM) components such as fibronectin, are bound to the integrin extracellular globular head domain whereas a multitude of cytoskeletal adaptor proteins interact via the cytoplasmic tail. Thus, integrins create a link between the ECM and the actin cytoskeleton. Ligand binding can be modulated at the level of integrin clustering (avidity) or by activation of integrins through conformational changes in the extracellular ligand-binding domains (affinity) (Calderwood, 2004; Takagi and Springer, 2002). For instance, binding to soluble ligands through integrin α5β1, αvβ3 or αIIbβ3 can be enhanced more than 20-fold by divalent cations or stimulatory antibodies, leading to firm adhesion of cells that show no appreciable adhesion to immobilized ligands in the absence of such stimuli (Danen et al., 1995; Mould et al., 1995; Smith et al., 1994). In turn, integrin-mediated adhesion can modulate various intracellular signaling cascades (Hynes, 2002). Following cell adhesion to the ECM, integrins and associated proteins assemble in cell-matrix adhesions, which organize the actin cytoskeleton. In epithelial cells, focal adhesions are randomly distributed across the ventral surface or along the cell border. In mesenchymal cells such as fibroblasts, they orient centripetally and concentrate in a few peripheral sites connecting long F-actin stress fibers, causing a typical elongated, contractile morphology.

Fibronectin is essential for embryonic development and is abundantly present in the ECM associated with wound healing and angiogenesis (Hynes and Zhao, 2000). Mesenchymal cells secrete compact and inactive soluble fibronectin dimers, which are assembled into insoluble fibronectin fibrils following their interaction with cell surface receptors of the integrin and syndecan families (Wierzbicka-Patynowski and Schwarzbauer, 2003). Fibronectin fibrils are assembled into a matrix that is important for anchorage of cells and guides cell migration during embryonic development and wound healing (Hynes and Zhao, 2000). Integrins α5β1, α8β1, αvβ1, αvβ3, αIIbβ3, αvβ5, αvβ6 and αvβ8 recognize the common integrin-binding motif Arg-Gly-Asp (RGD) that is found in many ECM components, including fibronectin (Danen and Sonnenberg, 2003). Of these integrins, α5β1 is particularly efficient in mediating fibronectin matrix assembly, although others can compensate for its absence to some extent (Wennerberg et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). Fibronectin fibrillogenesis requires extensive remodeling of cell-matrix adhesions (Pankov et al., 2000), and contractility of the actin-myosin cytoskeleton, which is stimulated by the small GTPase Rho through activation of myosin-II. Contractility drives fibronectin fibrillogenesis by creating sufficient tension to stretch the compact fibronectin dimers and expose intermolecular fibronectin-binding sites (Zhong et al., 1998).

Integrin-mediated cell adhesion triggers a rapid inactivation of the small GTPase RhoA (Ren et al., 1999), which is important to relieve contractility and allow cell spreading. As cell spreading ends, Rho-mediated cytoskeletal contractility gradually increases, which coincides with maturation of focal adhesions and initiation of

of αvβ3 in these processes using mouse embryonic fibroblasts (MEFs) isolated from *Itgb3*-knockout mice. The levels of GTP-RhoA in β3-integrin-null MEFs were similar to those in MEFs isolated from wild-type littermates, indicating that RhoA activation does not require αvβ3 integrin (Fig. 1B, supplemental material Fig. S1A). Similarly, fibronectin matrix assembly mediated by β3-integrin-null MEFs occurred equally efficiently as that mediated by wild-type MEFs (Fig. 1C). Ectopic expression of β3 integrin did not further stimulate RhoA activity, whereas increased expression of β1 integrin led to enhanced activation of RhoA (Fig. 1B). Increased expression of β1 integrins in β3-integrin-null MEFs also led to a more elongated, contractile cytoskeletal organization, whereas the presence or absence of β3 integrin did not affect morphology (Fig. 1D). Moreover, suppression of endogenous β1 integrins using *Itgb1*-specific siRNAs caused a phenotypic switch in wild-type MEFs characterized by increased cell spreading, more random distribution of focal adhesions and formation of cell-cell adhesions (Fig. 1E,F). We were unable to completely silence β1 integrin expression in MEFs and the level of suppression reached did not lead to detectable reduction of RhoA-GTP levels (not shown). Nevertheless, the conversion to a more epithelial morphology (strongly resembling that of β1-integrin-deficient GEβ3 cells; Fig. 1A) upon β1 integrin silencing was in complete agreement with our previous findings using β1-integrin-null cells. Silencing expression of β1 integrins in *Itgb3*-knockout MEFs caused cell rounding but the cells that remained attached resembled GE11 β1-integrin-knockout cells, growing in islands with extensive cell-cell contacts (Fig. 1E,F; compare with Fig. 3A, left image).

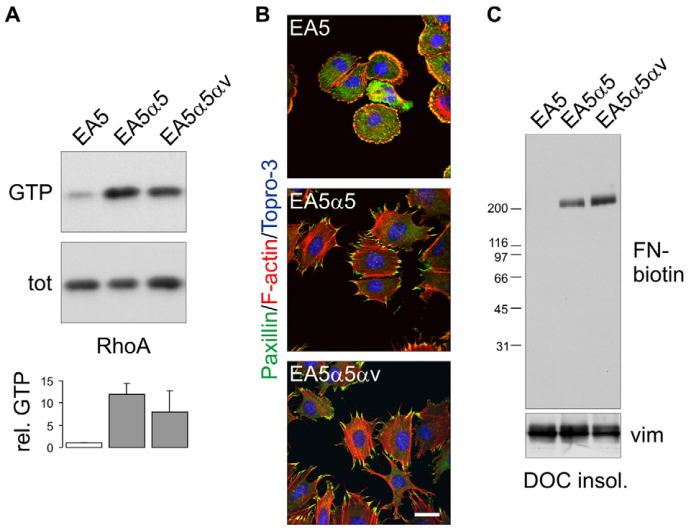

Fig. 2. Integrin α5 is required for efficient RhoA activation and fibronectin fibrillogenesis. (A) Western blot analysis of RhoA activity assay on lysates of EA5 cells expressing indicated integrins. Quantification shows relative RhoA activation ± s.d. compared with EA5 cells from two independent experiments. (B) EA5 cells expressing indicated constructs stained for paxillin (green) and F-actin (red), with the nucleus in blue. Scale bar: 10 μm. (C) Western blot analysis of assembled fibronectin-biotin and vimentin (loading control) in DOC-insoluble lysates of EA5 cells expressing indicated constructs. Locations of molecular size markers (in kDa) are indicated.

Of the β1 integrins, α5β1 integrin most efficiently supports fibronectin matrix assembly but integrins α8β1, α4β1 and αvβ1 can also mediate cell adhesion to immobilized fibronectin. To determine whether α5β1 integrin is the β1 integrin responsible for the typical fibroblast-like characteristics described above, we used differentiated *Itga5*-knockout ES cells (EA5). RhoA-GTP levels were very low in EA5 cells and restoring expression of α5 integrin induced efficient RhoA-GTP loading (Fig. 2A, supplementary material Fig. S1B). Expression of an α5αv integrin chimera, consisting of the α5 integrin extracellular and transmembrane domains and the αv integrin cytoplasmic domain similarly induced RhoA-GTP loading, indicating that α5-integrin-specific sequences in the cytoplasmic tail are not required for enhancing RhoA activation (Fig. 2A). The very low RhoA-GTP levels in EA5 cells were accompanied by a flat circular cell shape with dispersed focal adhesions. Expression of α5 integrin or α5αv integrin induced a reorganization of the cytoskeleton with long F-actin stress fibers connecting peripheral focal adhesions (Fig. 2B). Likewise, fibronectin fibrillogenesis was strongly enhanced in the presence of α5 integrin or α5αv integrin in EA5 cells (Fig. 2C), which is consistent with previous findings (Sechler et al., 1997; Wu et al., 1993).

Together, these results demonstrate that the typical fibroblast-like elongated, contractile morphology that is associated with high levels of Rho-GTP and fibronectin matrix assembly do not require αvβ3 integrin and are primarily stimulated by α5β1 integrin.

The extracellular ligand-binding domain of α5β1 integrin specifies fibroblast morphology, RhoA activation and fibronectin fibrillogenesis

In addition to the obvious essential role of the extracellular domain in ligand binding, the integrin transmembrane and cytoplasmic domains regulate ligand affinity and avidity by controlling integrin conformation and clustering (Calderwood, 2004; Li et al., 2003; Luo et al., 2007). The β1 integrin cytoplasmic domain is exchangeable with that of β3 integrin with no effect on Rho activation, fibronectin fibrillogenesis or fibroblast morphology when expressed in β1-integrin-deficient GE11 cells (Danen et al., 2002). To test whether specific amino acid residues in the transmembrane domain of β1 integrin are required for α5β1-integrin-mediated support of fibroblast morphology, Rho activity, and fibronectin fibrillogenesis, we generated a chimeric $\beta1^{e}3^{t+i}$ subunit, consisting of a β1 integrin extracellular domain and β3 integrin transmembrane and cytoplasmic domains. When expressed in β1-integrin-deficient GE11 cells (supplementary material Fig. S1C), this chimera promoted focal adhesion distribution and enhanced RhoA activity similar to wild-type β1 (Fig. 3A,B). Moreover, similarly to wild-type β1 integrin, $\beta1^{e}3^{t+i}$

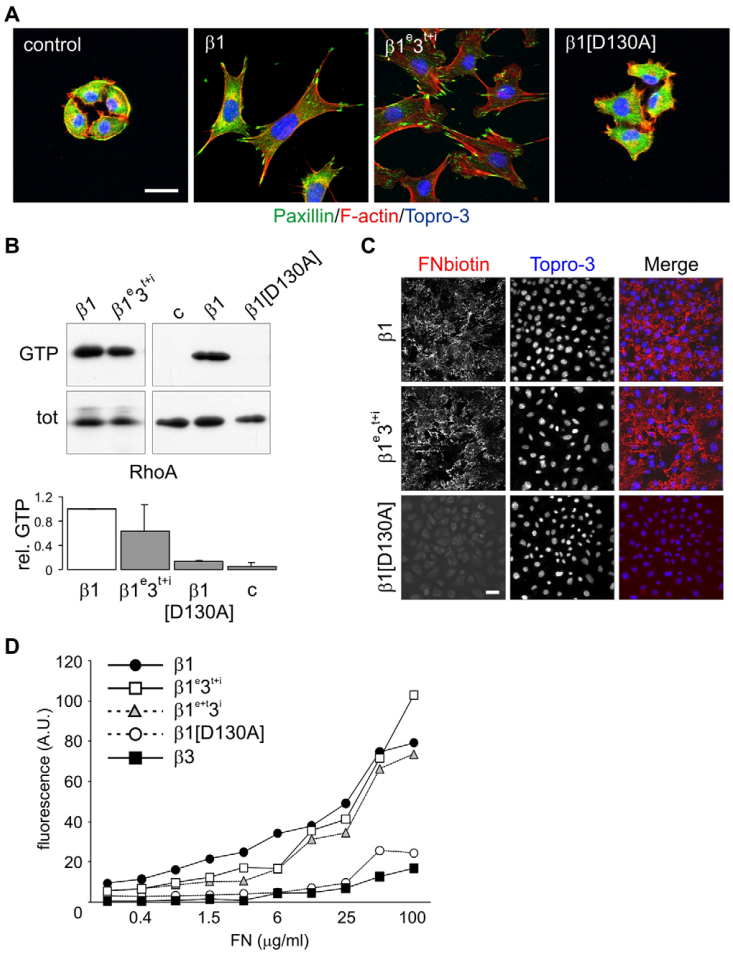

Fig. 3. Ligand binding to the extracellular domain of β1 integrin is required for RhoA activation and fibronectin fibrillogenesis. (A) GE11 cells expressing indicated β1 integrin subunits stained for paxillin (green), F-actin (red). Nuclei are stained blue with Topro-3. Scale bar: 10 μm. (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated β1 integrin subunits. Quantification shows relative RhoA activation ± s.d. compared with GEβ1 cells from two experiments. (C) Assembled fibronectin-biotin on GE11 cells expressing indicated β1 integrin subunits; fibronectin is red and nuclei are blue. Scale bar: 50 μm. (D) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-fibronectin.

efficiently supported fibronectin matrix assembly (Fig. 3C), arguing against a specific role of the transmembrane and cytoplasmic domains of β1 integrin in these processes. By contrast, expression of a β1[D130A] integrin subunit, containing a mutation in the extracellular I-like domain that abrogates ligand binding (Takada et al., 1992), failed to induce RhoA activation, a mesenchymal morphology, or fibronectin fibrillogenesis (Fig. 3, supplementary material Fig. S1C).

These findings indicate that the support of a fibroblast-like, contractile morphology associated with Rho activity and fibronectin fibillogenesis by α5β1 integrin is strictly dependent on ligand binding to its extracellular domain. Other regions of this integrin can be exchanged with corresponding regions of αvβ3 integrin without consequence and surface expression of α5β1 integrin without ligand interaction, which could affect (expression of) other surface receptors, is insufficient to support these processes.

The ability to bind to soluble fibronectin dimers correlates with RhoA activity and fibronectin fibrillogenesis

Many different integrins (including α5β1 integrin and αvβ3 integrin) can recognize the RGD motif and mediate cell adhesion to immobilized (stretched) fibronectin. However, we observed that all integrin β-subunits that supported a fibroblast-like cytoskeletal organization, Rho activity and fibronectin fibrillogenesis (β1, $\beta1^{e+t}3^{i}$ and $\beta1^{e}3^{t+i}$), efficiently bound to soluble (compact and inactive) fibronectin dimers whereas the other β-subunits tested (β3, β1[D130A]) did not (Fig. 3D). This suggested that differences in binding to soluble fibronectin between α5β1 integrin and αvβ3 integrin could explain not only the particular efficiency with which α5β1 integrin mediates fibronectin matrix assembly but also the different abilities of these integrins to support a fibroblast-like, contractile cytoskeletal organization and Rho activation.

We used mutants of β3 integrin, which, in the context of αIIbβ3, are locked in a low- or high-affinity conformation for the RGD sequence in fibrinogen (Luo et al., 2003; Luo et al., 2004), the integrin recognition sequence that is also present in the central cell-binding domain of fibronectin. Expression of a low-affinity β3[T329C; A347C] did not affect the poorly spread GE11 cell morphology (Fig. 4A center image; compare with Fig. 3A left image). Expression of the high-affinity β3[N305T] and β3[V332C; M335C] mutants stimulated cell spreading but distribution of focal adhesions in these cells was similar to those expressing wild-type β3 integrin (Fig. 4A; compare with Fig. 1A right image). In line with these morphological similarities, the high-affinity mutants failed to enhance RhoA-GTP levels or fibronectin fibrillogenesis in the absence of β1 integrin as also seen with wild-type αvβ3 integrin (Fig. 4B,C). As expected, based on findings in CHO cells (Luo et al., 2003; Luo et al., 2004) the β3[N305T] and β3[V332C; M335C] mutations strongly increased αvβ3-integrin-mediated binding of GE11 cells to soluble RGD (Fig. 5A). However, even in the presence of the activating divalent cation, manganese, locking αvβ3 integrin in a high affinity state for RGD failed to induce binding to soluble fibronectin (Fig. 5B,C). Again, α5β1 integrin efficiently bound soluble fibronectin in these experiments which could be competed with unlabeled fibronectin (Fig. 5B,C).

These results demonstrate that although RGD is a common recognition motif, affinity for RGD does not necessarily indicate binding to all RGD-containing ligands. The central cell-binding domain of compact soluble fibronectin dimers appears to be only available for binding to α5β1 and not to αvβ3 integrin. Notably, this indicates that efficient binding to soluble fibronectin dimers might in fact underlie the efficiency with which α5β1 integrin supports a fibroblast-like distribution of focal adhesions, contractile cell shape and Rho-mediated cytoskeletal contractility that drives fibronectin fibrillogenesis.

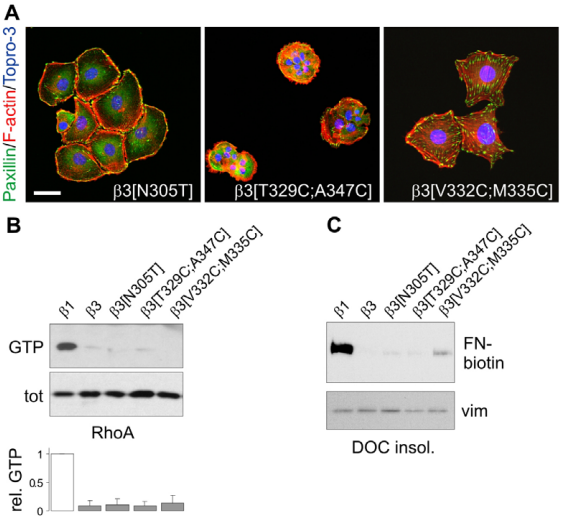

Fig. 4. High-affinity mutants of β3 integrin fail to stimulate RhoA activity and fibronectin fibrillogenesis. (A) Images of β1-integrin-deficient GE11 cells expressing indicated β3 integrin affinity mutants stained for paxillin (green), F-actin (red) and the nucleus (blue). Scale bar: 10 µm. (B) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated constructs. Quantification shows relative RhoA activation ± s.d. compared with GEβ1 cells from two experiments. (C) Western blot analysis of assembled fibronectin-biotin and vimentin (loading control) in DOC-insoluble lysates of GE11 cells expressing indicated constructs.

Specificity in the I-like domain controls binding of soluble fibronectin, focal adhesion distribution, Rho-mediated contractility and fibronectin matrix assembly independently of syndecan-4

Since ligand binding is required for α5β1 integrin to support a contractile, fibroblast-like morphology and Rho activity (β1[D130A]; Fig. 3) and since its ability to efficiently bind soluble fibronectin dimers appeared to underlie the difference between α5β1 integrin and αvβ3 integrin in this respect (Fig. 5), we analyzed a more subtle mutation in the I-like domain that participates in ligand binding. Exchanging the CTSEQNC hypervariable sequence in the I-like domain of β1 integrin with the corresponding region of β3 integrin has been shown to cause a shift in ligand specificity that leads to adhesion to vitronectin, von Willebrand factor and fibrinogen without affecting adhesion to immobilized (stretched) fibronectin (Takagi et al., 1997). We expressed such a β1-3-1 integrin subunit in β1-integrin-deficient GE11 cells (supplementary material Fig. S1D). Although adhesion to immobilized fibronectin was similar, cells expressing this chimera displayed strongly reduced binding of soluble fibronectin compared with cells expressing wild-type α5β1 integrin (Fig. 6A). Importantly, in addition to causing a marked inhibition of fibronectin matrix assembly, this was accompanied by a strong reduction in RhoA-GTP levels and by a random (epithelial-like) distribution of focal adhesions (Fig. 6B,C,D).

Rho-mediated contractility is required for fibronectin fibrillogenesis (Zhang et al., 1997; Zhong et al., 1998) and RhoA-GTP levels and fibronectin matrix assembly correlated for all the integrin constructs we tested. High RhoA-GTP levels were also associated with soluble fibronectin binding and fibroblast-like distribution of focal adhesions. We wondered (1) whether the high levels of RhoA-GTP were up- or downstream of soluble fibronectin binding to α5β1 integrin and the typical fibroblast-like distribution of focal adhesions and (2) whether fibronectin fibrillogenesis couples back to Rho activation in a positive feedback loop. To investigate these possibilities, binding of soluble fibronectin, fibronectin fibrillogenesis and RhoA activity were analyzed in cells expressing α5β1 under conditions where actomyosin contractility was blocked using ROCK or myosin-II inhibitors. In the presence of these inhibitors, fibronectin fibrillogenesis was strongly suppressed (ROCK) or even completely blocked (myosin-II), but binding of soluble fibronectin and RhoA-GTP levels were not affected (if anything they were slightly enhanced) (Fig. 7). These findings suggest that RhoA activity is downstream of fibronectin binding and they argue against a positive feedback loop from actomyosin contractility or fibronectin fibrillogenesis back to the regulation of Rho activity. Furthermore, the fact that the inhibitors ultimately led to disruption of focal adhesions (not shown) combined with the finding that silencing β1 in MEFs leads to a

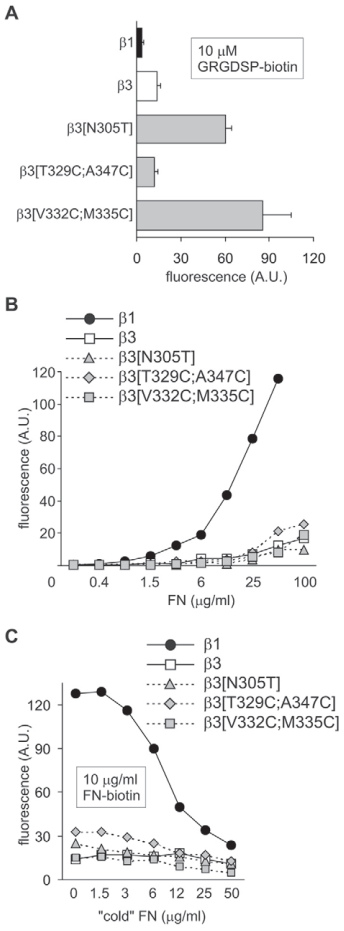

Fig. 5. Integrin binding of soluble GRGDSP and fibronectin. (A) Mean fluorescence ± s.d. demonstrating GRGDSP-biotin binding (10 μM) to indicated integrins expressed on GE11 cells analyzed by flow cytometry. (B) Mean fluorescence analyzed by flow cytometry, demonstrating binding of indicated integrins to different concentrations of soluble FITC-fibronectin. Binding to integrin β3 was shown previously in Fig. 3D. (C) Mean fluorescence demonstrating binding of soluble fibronectin-biotin (10 μg/ml) to indicated integrins upon competition with increasing concentrations of unlabeled fibronectin analyzed by flow cytometry.

more epithelial distribution of focal adhesions (Fig. 1F) while in both cases RhoA levels remain high, argues against the possibility that the fibroblast-like cytoskeletal organization acts upstream of RhoA.

It has been reported that the transmembrane proteoglycan syndecan-4 acts in concert with integrins to mediate fibronectin matrix assembly (Chung and Erickson, 1997) and can modulate the formation of cell-matrix adhesions and the activities of Rho-GTPases, including Rac1 (Bass et al., 2007) and RhoA (Dovas et al., 2006; Saoncella et al., 1999). We investigated whether syndecan-4 played a role in the support of fibroblast-like cytoskeletal organization, Rho activity or fibronectin fibrillogenesis by α5β1 integrin. For this purpose, we silenced syndecan-4 expression in GEβ1 cells using syndecan-4 specific siRNAs. A complete knockdown of surface expression of syndecan-4 was achieved within 48 hours, whereas syndecan-4 expression was unaffected in control siRNA transfected GEβ1 cells (Fig. 8A). The formation and distribution of cell-matrix adhesions and organization of the actin cytoskeleton remained unaltered in cells without syndecan-4 (Fig. 8B). Similarly, fibronectin matrix assembly and binding of soluble fibronectin by α5β1 integrin was still intact in GEβ1 cells lacking syndecan-4 (Fig. 8C,D). Finally, α5β1-integrin-supported RhoA activation was also unaffected by syndecan-4 silencing (Fig. 8E) although for this assay, cells had to be expanded to obtain sufficient lysate resulting in incomplete syndecan-4 downregulation (not shown). Notably, a knockdown of syndecan-4 expression levels in GEβ3 cells induced a dramatic morphological change, and GEβ3 cells lacking syndecan-4 were unable to form their typical flattened circular shape but instead formed an irregular cell border containing several cytoskeletal extensions (Fig. 8F,G). These experiments indicate that syndecan-4 can act in concert with αvβ3 integrin to regulate cell-matrix adhesion distribution when α5β1 integrin is absent but syndecan-4 is not required for cytoskeletal organization, focal adhesion formation/distribution, Rho activity or fibronectin matrix assembly in the presence of α5β1 integrin.

Taken together, our data demonstrate that following the initial drop in RhoA activity during cell adhesion, the ability of α5β1 integrin to bind compact soluble fibronectin dimers drives the typical fibroblast-like distribution of focal adhesions and the accumulation of Rho activity, which, in turn, stimulates fibronectin fibrillogenesis (Fig. 9). Interactions of fibronectin with αvβ3 integrin or syndecan-4 are dispensable for all these processes.

Discussion

Fibronectin fibrillogenesis requires integrin-mediated binding of soluble fibronectin dimers and depends on Rho-mediated cytoskeletal contractility to stretch integrin-bound fibronectin molecules exposing cryptic fibronectin-binding sites (Geiger et al., 2001; Mao and Schwarzbauer, 2005; Zhong et al., 1998). The α5β1 integrin mediates fibronectin matrix assembly with particular efficiency although other integrins can substitute for α5β1 integrin to some extent (Wennerberg et al., 1996; Wu et al., 1996; Yang and Hynes, 1996). Our current study indicates that α5β1 integrin not only promotes fibronectin matrix assembly, but also stimulates the contractile, fibroblast-like morphology. The ability of α5β1 integrin to efficiently bind compact soluble fibronectin dimers appears to drive the appropriate redistribution of focal adhesions to support this cell shape that is associated with increased RhoA activity, which, in turn, stimulates fibronectin fibrillogenesis. Such dynamic behavior of α5β1 integrin was previously implicated directly in fibronectin fibrillogenesis (Pankov et al., 2000). Our findings indicate that an analogous process underlies the distribution of focal adhesions leading to the typical contractile cell shape observed in fibroblasts. Integrins that cannot bind soluble fibronectin stimulate cell spreading and focal contact formation but these adhesions distribute randomly and are connected by short F-actin fibers, leading to an overall non-contractile, flat, epithelial-like cell shape. These findings point to

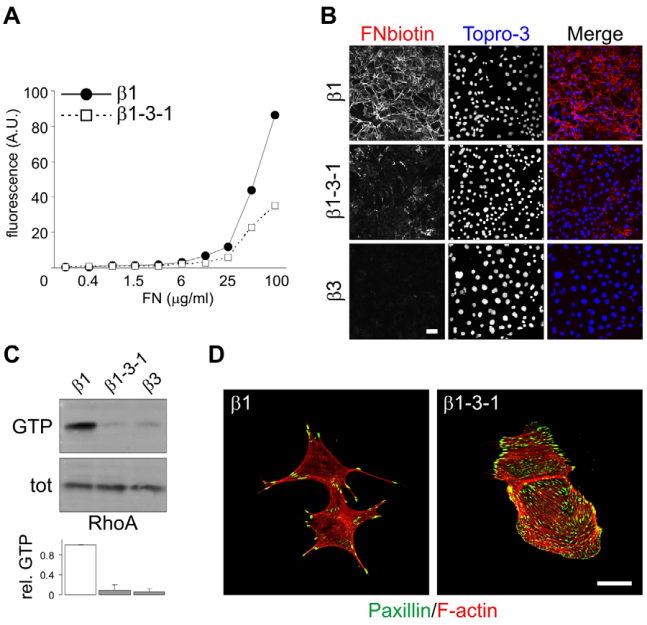

Fig. 6. High-affinity binding to the hypervariable region of the β1 I-like domain controls signaling to fibronectin fibrillogenesis. (A) Mean fluorescence analyzed by flow cytometry, demonstrating binding of integrin β1 and β1-3-1 to different concentrations of soluble FITC-fibronectin. (B) Images of assembled fibronectin-biotin on GE11 cells expressing β1 or β1-3-1; fibronectin is red and nucleus blue. Scale bar: 50 μm. (C) Western blot analysis of RhoA activity assay on lysates of GE11 cells expressing indicated integrins. Quantification shows relative RhoA activation ± s.d. compared with GEβ1 cells from two independent experiments. (D) Images of GE11 cells expressing β1 integrin or β1-3-1 stained for paxillin (green) and F-actin (red). Scale bar: 10 μm.

a critical role for α5β1 integrin as an efficient mechano-transducer, which couples binding of soluble fibronectin dimers to a cytoskeletal organization that supports the assembly of a fibronectin matrix (Fig. 9).

In agreement with our findings, α5β1 integrin has recently been demonstrated to support ROCK-mediated contractility in fibroblasts (Gaggioli et al., 2007; White et al., 2007). However, others have shown that overexpression of αvβ3 integrin can stimulate RhoA activity in leukocytes (Butler et al., 2003) and CHO cells (Miao et al., 2002). Notably, these cells express endogenous β1 integrins making it difficult to compare these studies with our own. In addition, multiple different cell surface receptors can regulate the activity of Rho-GTPases and may contribute to the difference between these studies. Nevertheless, in our previous studies we were unable to observe any stimulation of RhoA activity by αvβ3 integrin in the absence of β1 integrins (Danen et al., 2002; Danen et al., 2005). Here we use *Itgb3*-knockout MEFs to demonstrate directly that αvβ3 integrin is dispensable for RhoA activation. Wild-type and β3-integrin-null MEFs also display the same fibroblast-like cytoskeletal organization. Moreover, a strong RNAi-mediated reduction in the expression of β1 integrins in MEFs produces a cytoskeletal organization that is very similar to that in β1-deficient cells expressing high levels of αvβ3 integrin (GEβ3). Thus, although expression of β1 integrin induces a morphological switch that resembles an 'epithelial-to-mesenchymal transition' in β1-integrin-null cells (Danen, 2002), silencing β1 integrin in MEFs induces what appears like a 'mesenchymal-to-epithelial transition'.

We observe a tight correlation between high RhoA-GTP levels and focal adhesion dynamics, contractile morphology, and fibronectin matrix assembly (Danen 2002; Danen, 2005) (this study). Experiments using inhibitors argue against a positive feedback loop from cytoskeletal organization to RhoA GTP loading but we do not know at present how the ability of α5β1 integrin to bind soluble fibronectin supports the activity of RhoA. Recently, the guanine nucleotide exchange factors (GEFs) Lsc and LARG were shown to link cell adhesion on fibronectin to RhoA GTP loading and focal adhesion and stress fiber formation (Dubash et al., 2007). However, the integrins tested in our study that do not bind soluble fibronectin and do not stimulate RhoA activity do in fact efficiently support the formation of focal adhesions and short stress fibers. RhoA-mediated contractility appears to be more important for the distribution of focal adhesions and orientation of stress fibers. If regulation of GEFs and GAPs (GTPase-activating proteins) is involved, our experiments using chimeric integrins indicate that obligate interactions of such proteins or their regulators with α5β1-integrin-specific residues in the cytoplasmic domains do not underlie α5β1-integrin-stimulated RhoA activation. However, it is possible that binding of soluble fibronectin to α5β1 integrin induces conformational alterations and/or clustering of the integrin that affects the localization and/or activity of such regulatory proteins.

Syndecan-4 can act in concert with integrins to regulate cytoskeletal organization during cell adhesion to fibronectin through interactions at the HepII domain in fibronectin. Using an RNAi approach we rule out the idea that crosstalk with syndecan-4 is involved in the fibroblast-like cytoskeletal organization, Rho activity and fibronectin matrix assembly that is supported by α5β1 integrin. Our findings do not seem to support the previously reported role of syndecan-4 in stimulation of RhoA-mediated processes (Saoncella et al., 1999). However, others have recently shown that during adhesion to fibronectin, syndecan-4 is required for regulation of Rac1, but not RhoA (Bass et al., 2007). This might also explain why syndecan-4 knockdown strongly affects cytoskeletal

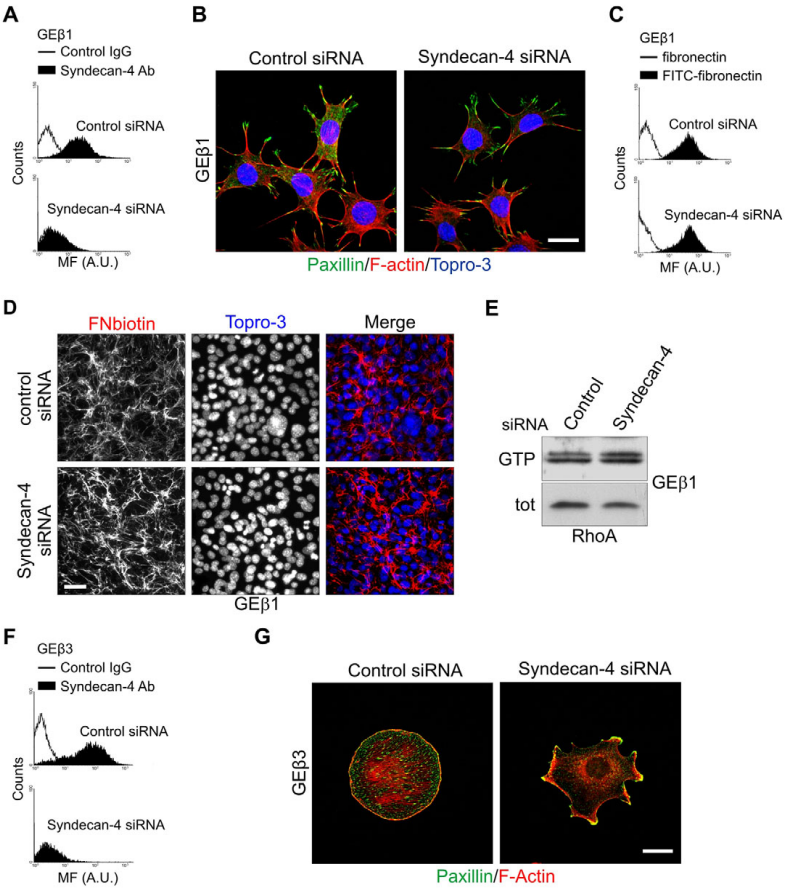

Fig. 8. Syndecan-4 is not required for α5β1-integrin-supported RhoA signaling. (A) Flow cytometry analysis of endogenous syndecan-4 surface expression on GEβ1 cells transfected with syndecan-4 or control siRNA. (B) Images of GEβ1 cells transfected with syndecan-4 or control siRNA stained for paxillin (green), F-actin (red) and the nucleus (blue). (C) Flow cytometry analysis of soluble FITC-fibronectin (10 µg/ml) binding to GEβ1 cells with or without syndecan-4 knockdown. (D) Images of assembled fibronectin-biotin on GEβ1 transfected with syndecan-4 or control siRNA; fibronectin is red and nucleus is blue. Scale bar: 50 µm. (E) Western blot analysis of RhoA activity assay on lysates of syndecan-4 knockdown and control GEβ1 cells. (F) Flow cytometry analysis of endogenous syndecan-4 surface expression on GEβ3 cells transfected with syndecan-4 or control siRNA. (G) Images of GEβ3 cells transfected with syndecan-4 or control siRNA stained for paxillin (green) and F-actin (red).

et al., 2002). cDNA encoding human α5 (provided by Erkki Ruoslahti, Cancer Research Center, The Burnham Institute, La Jolla, CA) was cloned into LZRS-neo. To generate a cDNA encoding a chimeric human α5αv integrin subunit, a fragment containing the extracellular and transmembrane domain of α5 integrin was digested from LZRS-α5 as a *Swa*I/*Hin*dIII fragment using the internal *Hin*dIII site immediately downstream of the transmembrane region. This was ligated to a *Hin*dIII/*Sna*BI cDNA fragment derived from LZRS-αv by PCR amplification of the αv integrin cytoplasmic domain in which the *aggatg* sequence immediately upstream of the cytoplasmic tail of αv integrin was changed to *aagctt*, creating a *Hin*dIII site. To generate a cDNA encoding a chimeric human $\beta1^{c3^{tri}}$ subunit, the *Itgb1* sequence downstream of the internal *Sna*BI site (70 nucleotides upstream of the transmembrane region) was replaced with the corresponding sequence derived from LZRS-αv by PCR amplification in which the *ccagta* sequence was changed to *tacgta*, creating a *Sna*BI site. These cDNAs, as well as cDNAs encoding β3[N305T], β3[T329C;A347C] and β3[V332C;M335C] (provided by Bing-Hao Luo and Timothy A. Springer, CBR Institute for Biomedical Research, Boston, MA) and cDNA encoding β1[D130A] (provided by Yoshikazu Takada, University of California Davis Medical Center, Sacramento, CA) were recloned into LZRS-neo. The β1-3-1 expression plasmid was provided by Yoshikazu Takada. Retroviral constructs were transfected into ecotrophic packaging cells to generate virus-containing culture supernatants. β3 knockout MEFs, EA5 and GE11 cells were transfected with cDNA using effectene (Qiagen) or transduction with retroviral supernatants and positive cells were bulk sorted at least twice by FACS for the human integrin expressed.

Antibodies and other materials

Monoclonal antibodies used were anti-human α5 NKI-Sam1 (provided by Carl Figdor, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands), anti-human β1 TS/2/16, clone 18 (Transduction Laboratories), anti-human β3 C17 (provided by Ellen van der Schoot, Sanquin, Amsterdam, The Netherlands), anti-

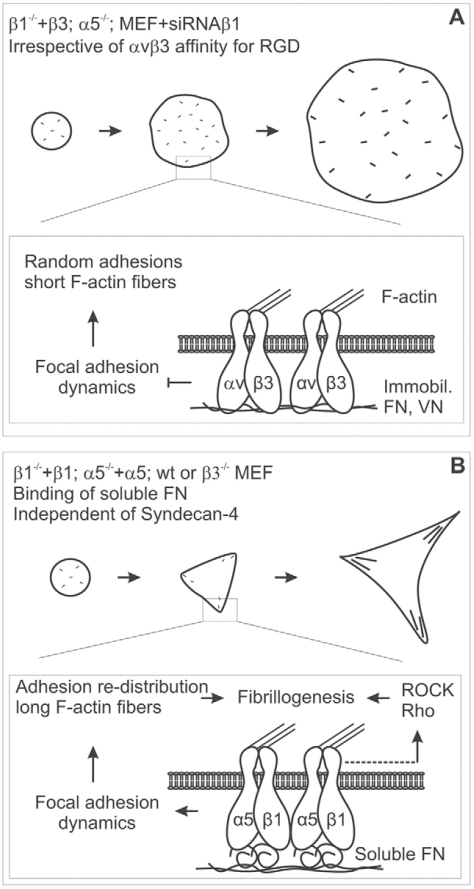

Fig. 9. A model for the role of α5β1 integrin in reorganization of focal adhesions, contractility, cell morphology and fibronectin-matrix assembly. (A) Adhesion to immobilized (stretched) RGD-containing ECM components such as vitronectin (VN) or fibronectin (FN) through αvβ3 integrin in the absence of α5β1 integrin promotes the formation of randomly distributed cell-matrix adhesions leading to an epithelial-like, flat, circular morphology. (B) In the presence of α5β1 integrin, binding of compact soluble fibronectin dimers to this integrin promotes the dynamic centripetal redistribution of focal adhesions. Together with the high activity of RhoA that is supported in the presence of α5β1 integrin, this allows the cytoskeletal organization that drives fibronectin fibrillogenesis.

mouse β3 (clone 2C9.G2, Pharmingen), anti-paxillin (clone 349, BD Transduction Laboratories), anti-RhoA (clone 26C4, Santa Cruz), anti-Syndecan-4 (clone KY/8.2, Pharmingen), anti-vimentin (clone K36, provided by F. Ramaekers, University of Maastricht, Maastricht, The Netherlands). Texas-Red-conjugated phalloidin and Topro-3 were purchased from Molecular Probes. Texas-Red-conjugated streptavidin was purchased from Pierce Chemical Co. Human plasma fibronectin and biotinylated-fibronectin were prepared as described previously (Danen et al., 2002). GRGDSP peptide was generated at the Netherlands Cancer Institute and biotinylated using EZ-link Sulfo-NHS-Biotin (Pierce Chemical Co.) according to the manufacturer's protocol. Y27632 and blebbistatin were obtained from Calbiochem.

RhoA activity assays

Cells were plated overnight to subconfluency before lysis in Nonidet P-40 lysis buffer [0.5% Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, supplemented with a protease inhibitor mix (Sigma-Aldrich)], and lysates were clarified by centrifugation at 20,000 ***g*** for 20 minutes at 4°C. A 1% aliquot was removed for determination of total quantities of RhoA. Clarified lysates were then incubated for 45 minutes at 4°C with a GST fusion protein of the Rho-binding domain of the Rho effector protein Rhotekin (Ren et al., 1999). Complexes were bound to glutathione-conjugated beads, and washed three times in Nonidet P-40 lysis buffer. The samples were analyzed by SDS-PAGE and western blotting.

Immunofluorescence and flow cytometry

For immunofluorescence, cells were fixed in 2% paraformaldehyde, permeabilized in 0.4% Triton X-100, blocked with 2% BSA, and incubated with anti-paxillin antibody, followed by FITC-labeled secondary antibody, phalloidin-Texas-Red and Topro-3 staining. To visualize fibronectin fibrillogenesis, cells were plated on fibronectin-coated coverslips for 4 hours and subsequently incubated for an additional 20 hours in medium containing 10% fibronectin-depleted serum supplemented with 10 μg/ml biotinylated fibronectin. Cells were fixed in 2% paraformaldehyde, blocked with 2% BSA and stained with streptavidin-Texas-Red. Subsequently, coverslips were permeabilized in 0.4% Triton X-100 and stained with Topro-3. Preparations were mounted in Mowiol 4-88 solution supplemented with DABCO (Calbiochem) and analyzed using a confocal Leica TCS-NT microscope. Images were obtained using a 40× or 63× oil objective and imported into Adobe Photoshop.

For flow cytometry of integrin expression and cell sorting, cells were trypsinized, collected in culture medium, washed with PBS, and incubated with primary antibodies in PBS containing 2% serum for 1 hour at 4°C. For flow cytometry of syndecan-4 expression, cells were harvested using enzyme-free Dissociaton buffer (Gibco), washed with 0.1% BSA/0.1% sodium azide followed by primary antibody incubation. Cells were then washed in PBS, incubated with FITC-, PE- or APC-conjugated secondary antibodies for 1 hour at 4°C, washed in PBS, and analyzed on a FACSCalibur or sorted on a FACStar plus® (Becton Dickinson).

DOC insolubility assays

Cells were labeled with biotinylated fibronectin as described above and lysed in DOC buffer [1% sodium deoxycholate (DOC), 20 mM Tris-HCl pH 8.5, 2 mM *N*-ethylmaleimide, 2 mM iodoacetic acid, 2 mM EDTA and 2 mM PMSF]. Lysates were passed through a 23 GA needle and DOC-insoluble material was collected by centrifugation at 20,000 ***g*** for 20 minutes at 4°C. The pellet was washed once with DOC buffer, resolved in reduced sample buffer and analyzed by SDS-PAGE and western blotting.

RGD and fibronectin binding assays

Cells were harvested, resuspended in DMEM supplemented with 0.5% BSA and 2 mM $MnCl_2$, and incubated with 10 μM biotinylated GRGDSP-peptide or 10 μg/ml biotinylated human plasma fibronectin for 1 hour at 4°C in the absence or presence of increasing concentrations of unlabeled fibronectin. The cell pellet was washed in 0.9% NaCl and 2 mM $MnCl_2$ and subsequently labeled with PE-conjugated streptavidin for 30 minutes at 4°C. To monitor dose-responsive fibronectin binding, cells were incubated with different concentrations of FITC-conjugated human plasma fibronectin for 1 hour at 4°C. Binding of the RGD-peptide or fibronectin was determined by flow cytometry.

siRNA transfection

Cells were plated at 40% confluency and were transfected the following day using DharmaFECT 1 reagent and a final concentration of 100 nM of integrin β1 SMARTpool siRNA (M-040783-00), Syndecan-4 ON-TARGET plus SMARTpool siRNA (L-044221-00), or siCONTROL non-targeting siRNA#2 (D-001210-02) purchased from Dharmacon. After replating, the cells were analyzed 48 hours post-transfection for integrin β1 or syndecan-4 surface expression, and used for immunofluorescence, RhoA activity assays and fibronectin binding assays.

We thank Richard Hynes for kindly providing Itgb3 knockout mice and ITGa5 knockout ES cells and Carl Figdor, Bing-Hao Luo, Frans Ramaekers, Erkki Ruoslahti, Timothy Springer, Yoshikazu Takada and Ellen van der Schoot for providing plasmids and antibodies. This work was supported by grant UL2006-3521 from the Dutch Cancer Society.

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Chapter 5

Integrins: Signaling Disease, and Therapy

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Integrins: Signaling, disease, and therapy

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Abstract

Background: Integrins are a family of transmembrane receptors that mediate cell-cell and cell-matrix adhesion. They are involved in stable cell adhesion and migration of cells. In addition, integrin-mediated interactions modulate the response to most, if not all growth factors, cytokines, and other soluble factors.

Purpose: In this review, we briefly explain how integrins can affect the multitude of signal transduction cascades in control of survival, proliferation, and differentiation. Subsequently, we primarily focus on targeting integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$ in disease and we discuss how antagonists of these integrins, including disintegrins, RGD peptides, small molecules, and function blocking antibodies, may be of therapeutical value either alone or, especially in the treatment of cancer, in combination with existing therapeutical strategies.

Keywords: *Adhesion, treatment, cancer*

Introduction

Integrins are cell surface receptors that mediate interactions with the extracellular matrix (ECM) or with counter-receptors on other cells. They cluster and recruit a large multi-protein complex to cell-ECM or cell-cell junctions, which connects them to the cytoskeleton. In addition, signaling proteins and their substrates accumulate at these sites, which regulate the stability of the adhesions and control cytoskeletal dynamics. Besides their critical role in stable cell adhesion and cell migration, integrin-mediated interactions modulate signaling by various other receptors including receptor tyrosine kinases (RTK), G-protein-coupled receptors, cytokine receptors, and others. Consequently, integrins play important roles in survival, proliferation, and differentiation. They are also implicated in several human diseases and integrin antagonists have been tested in preclinical models for various diseases including inflammation, thrombosis, arthritis, and cancer and some have even entered clinical trials. For the treatment of cancer, the expectation is that these antagonists may increase the efficacy of radio- and chemotherapy.

Integrins

Integrins are heterodimeric transmembrane receptors that bind with their globular head domain to components of the ECM. Some integrins can also bind counter receptors present on other cells, bacterial polysaccharides, or viral coat proteins. Intracellularly, integrins are connected via associated proteins to the actin cytoskeleton. 18 α and 8 β subunits are encoded in the human genome from which 24 different functional integrins are currently known to be generated (van der Flier & Sonnenberg 2001, Hynes 2002). Ligand binding can be regulated through integrin clustering and through modulation of the activity of individual integrins which involves the propagation of conformational changes from the cytoplasmic tails across the membrane towards the ligand-binding region (Liddington & Ginsberg, 2002). Integrins can also activate intracellular signal transduction cascades, a process referred to as 'outside-in signaling'. Integrin-mediated cell adhesion can trigger calcium fluxes, activate tyrosine and serine/threonine protein kinases and inositol lipid metabolism, and regulate the activity of the Rho family of small GTPases (Danen & Yamada 2001).

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Genetic studies in flies, worms, and mice have established important roles for integrins and integrin-associated proteins in the development and maintenance of tissues and in the progression of diseases (De Arcangelis & Georges-Labouesse 2000, Bouvard et al. 2001, Bokel & Brown 2002).

Integrin signaling

Integrins generally contain a short cytoplasmic domain which is devoid of enzymatic activity. Therefore outside-in signaling by integrins largely depends on interactions with neighbouring receptors, adaptor and signaling proteins.

Nevertheless integrin signaling is critically important for regulation of signal transduction pathways through distinct mechanisms (Figure 1):

(1) Integrins and growth factor receptors may activate parallel pathways that synergize at the level of activation of downstream signaling proteins. In this way threshold levels in signaling pathways can be lowered considerably (Chen et al. 1996, Renshaw et al. 1997).

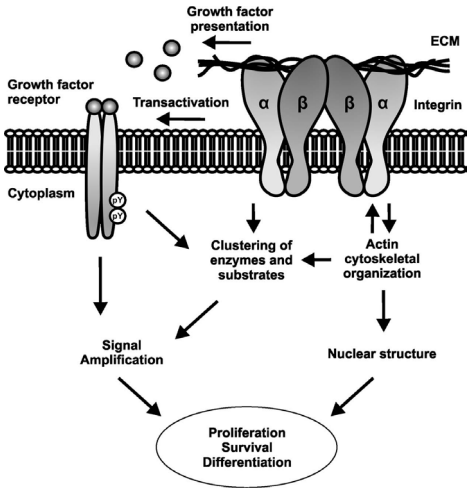

Figure 1. Cross-talk between growth factor receptors and integrins. Integrins affect signal transduction pathways through distinct mechanisms: (1) Together with growth factor receptors, integrins activate parallel pathways that synergize at the level of downstream signaling proteins; (2) Integrins initiate clustering of proteins in cell-matrix adhesions, thereby bringing kinases and substrates in close proximity; (3) Those cell-matrix adhesions also anchor the actin cytoskeleton and thereby generate cytoskeletal tension that affects the nuclear shape and gene expression; (4) Integrin-mediated adhesion can cluster and transactivate receptor tyrosine kinases, and (5) integrins organize the extracellular matrix and thereby regulate upstream signaling of receptors. See text for additional details.

(2) Cell-matrix adhesion initiates clustering of integrins in the plane of the membrane and reorganization of the actin cytoskeleton, which further stimulates the organization of integrins and associated proteins into large multi-protein platforms like focal adhesions, hemidesmosomes or podosomes. In those platforms integrins may increase signals generated by growth factor receptors by bringing kinases and substrates in close proximity (Burridge & Chrzanowska-Wodnicka 1996, Geiger et al. 2001).

(3) Cell-matrix adhesions act as anchoring sites for the actin cytoskeleton and as such they allow the generation of tension and shape changes. Via cytoskeletal connections with the nucleus such changes can affect the nuclear shape and chromatin structure which might explain the profound effect of integrin-mediated cell adhesion on the expression of genes (Maniotis et al. 1997, Lelievre et al. 1998).

(4) Integrin-mediated adhesion can cluster and transactivate several RTK including platelet-derived growth factor receptor (PDGFR), epidermal growth factor receptor (EGFR), Ron, Met, and vascular endothelial growth factor receptor (VEGFR) (Yamada & Even-Ram 2002) and members of the Src family kinases (Shattil 2005).

(5) Integrins regulate RTK signaling further upstream through their ability to organize the ECM. Proteoglycans in the ECM can bind and structurally modify various growth factors and integrin-mediated cell adhesion then allows their subsequent presentation to growth factor receptors (Faham et al. 1998). Through these mechanisms integrins play an essential role in regulating signaling pathways in control of survival, proliferation, and differentiation both in health and disease.

Survival

Most adherent cell types depend on integrin-mediated adhesion for survival (Giancotti & Ruoslahti 1999, Cordes 2006, Gilcrease 2007). Loss of adhesion causes cells to undergo apoptosis, a process referred to as anoikis (Frisch & Screaton 2001). Likely, anoikis is important to maintain the integrity of tissues by preventing cells from growing at inappropriate sites after losing adhesion from their original surrounding. Integrin-mediated cell adhesion in 2-dimensional culture systems stimulates phosphatidylinositol-3-kinase (PI3K)-mediated protein kinase B (PKB/AKT) activity and B-cell leukemia-2 (Bcl-2) expression which mediates survival signals (Giancotti & Ruoslahti 1999). In absence of serum factors

integrin-mediated adhesion to fibronectin enhances survival by activating c-Jun N-terminal kinase (JNK) in a focal adhesion kinase (FAK) dependent manner (Almeida et al. 2000). Integrin $\alpha 6\beta 4$ ligation also supports nuclear factor κB (NFκB)-mediated survival signals in 3-dimensional cultures of mammary epithelial cells (Weaver et al. 2002). On the other hand, integrins that are not ligand-bound can trigger apoptosis of fully adherent cells by recruitment and activation of caspase-8 suggesting that a given integrin expression profile renders a cell dependent on a specific ECM environment for its survival (Varner et al. 1995, Stupack et al. 2001).

Proliferation

The ability to grow in the absence of cell adhesion is a key property of oncogenically transformed cells. In normal untransformed cells, integrin-mediated cell adhesion regulates the G1 phase of the cell cycle (Assoian & Schwartz 2001). Integrins cooperate with RTK to stimulate the cyclin E/cyclin dependent kinase 2 (cdk2) activity that drives S-phase entry. Multiple different pathways have been described to connect integrins to cell cycle progression. Regulation of cyclin D1 expression, both at the level of gene transcription and protein accumulation, is a key element of the control of cell cycle progression by RTK and integrins. Control of extracellular signal-regulated kinase (ERK) activation can largely explain the transcriptional regulation of cyclin D1 by integrin-mediated adhesion.

Mitogen-stimulation of RTK and integrin-mediated adhesion can each independently stimulate ERK activation. However, only when adherent cells are stimulated with mitogens ERK activity is strong and sustained due to convergence of RTK and integrin signaling at the level of Raf or MAP/ERK kinase (MEK) (Chen et al. 1996, Renshaw et al. 1997).

There are several ways through which integrins can regulate ERK activity (Howe et al. 2002):

(1) Binding of integrins to the ECM stimulates the formation of an active FAK/Src signaling complex at sites of adhesion. Autophosphorylation of FAK at Tyr^{397} following integrin-mediated adhesion creates a binding site for the Src homology 2 (SH2) domain of Src (Schlaepfer & Hunter 1998). Subsequently, Src can phosphorylate other Tyr residues of FAK thereby creating binding sites for downstream effectors. Direct binding of growth factor receptor binding protein-2 (Grb2) to the active FAK/Src complex or indirect binding through Shc stimulates the Grb2-Sos-Ras-Raf-MEK-ERK pathway. Alternatively, Src can phosphorylate the scaffolding protein p130Cas (Crk associated substrate) that is also associated with FAK via its SH3 domain, thereby creating a binding site for the adaptor protein Crk. Either through association with son of sevenless (Sos) or through association with C3G (a guanine-nucleotide exchange factor for the small GTPase Rap-1), the interaction with Crk can result in ERK activation. Integrin-mediated adhesion also stimulates the association of the adaptor protein Nck with p130Cas, creating yet another potential link from p130Cas to ERK activation. Finally, PI(3)K can associate with phosphorylated Tyr^{397} in FAK and it may become activated upon integrin-mediated cell adhesion. PI(3)K may activate ERK through its role as a protein kinase or through modulation of Sos activity via its production of phosphatidylinositol-3,4,5-trisphosphate (PtdInsP3).

(2) Secondly, certain integrin α-subunits are coupled to the Src family kinase Fyn through association with the oligomeric transmembrane protein Caveolin-1 (Guo & Giancotti 2004). Upon integrin ligand binding, Fyn is activated and it subsequently recruits and phosphorylates Shc, creating a link to the Grb2-Sos-Ras-Raf-MEK-ERK pathway.

(3) Finally, integrin-mediated adhesion activates protein kinase C (PKC) and several PKC isoforms can directly activate Raf. Enhanced levels of phospholipids probably can explain the activation of PKC upon integrin-mediated adhesion. Also integrin-mediated adhesion leads to the activation of the p21-activated protein kinases (PAK) through several mechanisms, and PAK can activate both Raf and MEK.

Besides controlling ERK activity, suppression or relocalization of the cyclin dependent kinase inhibitors p21 and p27 by integrin-mediated adhesion also contributes to G1 cell cycle progression. Additionally, integrin-mediated adhesion increases expression of c-Myc through activation of c-Src (Benaud & Dickson 2001). The organization of the actin cytoskeleton by integrins is essential for adhesion-regulated proliferation and integrin-mediated control of the activity of Rho GTPases (enzymes critically involved in actin cytoskeletal organization) is an important aspect of adhesion-mediated regulation of the levels of cyclin D1 and cdk-inhibitors.

Differentiation

Integrin-mediated cell adhesion also regulates the expression of genes related to differentiation. Adhesion to basement membrane components stimulates the synthesis of milk proteins by increasing

phosphorylation of the prolactin receptor in cultured mammary epithelial cells (Li et al. 1987, Edwards et al. 1998). Integrin-mediated adhesion also primes monocytes for inflammatory responses (Haskill et al. 1988, Shi & Simon 2006). Another example of regulation of differentiation by integrins is the inhibition by integrin-blocking antibodies of the formation of contracting myotubes and expression of meromyosin by embryonic myoblasts (Menko & Boettiger 1987). Deletion of β1 integrins in embryonic stem (ES) cells showed that β1 is important for normal *in vitro* cardiac and myogenic differentiation, whereas neuronal differentiation is accelerated in β1-deficient ES cells (Fassler et al. 1996, Rohwedel et al. 1998). Finally, terminal differentiation of cultured keratinocytes under semi-solid conditions is inhibited by the integrin-ligand fibronectin or by adhesion-blocking antibodies to β1 integrins (Watt 2002). Moreover, a tumor-associated mutation in β1 was recently found that increases ligand binding and prevents terminal differentiation of keratinocytes which might contribute to the formation of epidermal neoplasia (Evans et al. 2003).

Integrins in disease

Aberrant cell adhesion and migration have been implicated in several diseases, including a number of inflammatory disorders such as rheumatoid arthritis, inflammatory bowel disease and asthma, as well as cardiovascular diseases, thrombosis, and cancer. This often correlates with alterations in the expression or functionality of integrins. For instance, deletion of the $\alpha 6\beta 4$ integrin leads to a skin blistering disease termed Epidermolysis bullosa (Borradori & Sonnenberg 1999), deletion of $\alpha 7\beta 1$ causes congenital muscular dystrophy (Vachon et al. 1997), and in patients with Glanzmann's Thrombasthenia platelets fail to aggregate, due to quantitative or qualitative defects of αIIbβ3 (Hodivala-Dilke et al. 1999). On the other hand, in osteoporosis up-regulation of integrin $\alpha v\beta 3$ causes enhanced bone resorption by osteoclasts (Lakkakorpi et al. 1993) and high expression levels of various types of integrins have been correlated with tumor progression in a numbers of cancers (Mizejewski 1999).

The use of integrin antagonists

Integrin-blocking strategies have been developed to treat a large number of diseases (Table I). Integrin antagonists comprise small molecule compounds, peptidomimetics, and monoclonal antibodies (mAb). We will discuss use and action of several drugs with focus on cancer and antagonists of the integrins $\alpha 5\beta 1$ and $\alpha v\beta 3$, being the most extensively studied targets in this disease.

Anti-angiogenesis is an emerging approach for cancer treatment. Angiogenesis, which is the formation of new blood vessels, is a vital process for tumor progression (Folkman 1971). Inhibition of new blood vessel formation has been shown to block the growth and spread of solid tumors in various animal models. There is substantial evidence that several integrins, including $\alpha v\beta 3$, $\alpha v\beta 5$, and $\alpha 5\beta 1$ have an important role in tumor angiogenesis (Brooks et al. 1994a, Friedlander et al. 1995, Kim et al. 2000). The regulation of cell migration and survival of endothelial cells during angiogenesis and metastasis via these integrins makes them suitable targets for anti-angiogenic therapy.

Disintegrins and RGD peptides

The RGD sequence is an important cell attachment recognition site for integrins in many ECM components (Pierschbacher & Ruoslahti 1984, Gardner & Hynes 1985, Plow et al. 1985) and has been used as a pharmaceutical application to treat aberrant cell adhesion related-diseases. Disintegrins are RGD-containing cysteine-rich peptides discovered in snake venoms (Gould et al. 1990). Some disintegrins specifically bind to integrin $\alpha v\beta 3$ and are applied as therapeutic agents for angiogenesis-dependent tumor growth and metastasis (Huang 1998). Others, such as Contortrostatin, which is a disulfide-linked homodimer of 13.5 kDa containing two RGD sites isolated from the venom of *Agkistrodon contortrix*, can bind $\alpha v\beta 3$, $\alpha v\beta 5$, as well as $\alpha 5\beta 1$ and have been shown to inhibit tumor growth and angiogenesis in an orthotopic xenograft model for breast cancer (Swenson et al. 2005).

The disadvantage of natural occurring peptides such as disintegrins is their relatively large size and low metabolic stability, which limits their usefulness for clinical application (McLane et al. 2004, Cai & Chen 2006). RGD peptides have been further optimized by incorporation of D-amino acids and use of cyclic structures. Cyclic RGD-containing pentapeptides are the most commonly used RGD-based $\alpha v\beta 3$ antagonists (Ruoslahti 1996). Cyclo (RGDfV) is a highly effective $\alpha v\beta 3$ antagonist with anti-tumor and anti-angiogenic effects (Brooks et al. 1994b, Friedlander et al. 1995). In a xenograft model for melanoma cyclo (RGDfV)-treatment hindered tumor growth and histological analysis indicated that the effect results from angiogenesis inhibition rather than inhibition of tumor cell $\alpha v\beta 3$ (Dechantsreiter et al. 1999). Systematic modification of this peptide resulted in a more active and selective compound named c(RGDf(NMe)V) also known as Cilengitide (EMD 121974) (Goodman et al. 2002). Cilengitide induces apoptosis in Glioblastoma and

Table I. Integrin antagonists and its targeted diseases.

Integrin target	Drug	Company	Disease	Reference
$\beta 2$	Efalizumab (RabTIVA®)	Genetech	Psoriasis	(Lebwohl et al. 2003)
$\alpha 4$	Natalizumab (Tysabri®)	Biogen Idec & Elan	MS, Crohn's disease	(Miller et al. 2003, Ghosh et al. 2003)
$\alpha 4\beta 1/\alpha 4\beta 7$	TR14035	N/A	Asthma	(Cortijo et al. 2006, Sircar et al. 2002)
$\alpha 4\beta 7$	MLN02	Millennium pharmaceuticals	Ulcerative colitis	(Feagan et al. 2005)
$\alpha 5\beta 1$	Volociximab (M200/Gemzar®)	PDL Biopharma Inc	Renal cell carcinoma, metastastic melanoma, pancreatic cancer	www.clinicaltrials.gov
	Endostatin™	EntreMed Inc.		(O'Reilly et al. 1997)
	ATN-161	N/A		(Stoeltzing et al. 2003, Livant et al. 2000)
$\alpha v\beta 3$	Chimeric 7E3 Fab (Abciximab/ReoPro®)	Centocor		(Trikha et al. 2002)
	Contortrostatin	N/A		(Clark et al. 1994, Trikha et al. 1994)
	C(RGDf(NMe)V) (Cilengitide, EMD 121974)	Merck KGaA	Angiogenesis, cancer	www.cancer.gov/clinicaltrials (Albert et al. 2006, Taga et al. 2002)
	C (RGDfV) cyclo (Arg-Gly-Asp-D-Phe-Val)	N/A		(Allman et al. 2000, Friedlander et al. 1995, Brooks et al. 1994b, Dechantsreiter et al. 1999)
	S247	Pharmacia Corp		(Reinmuth et al. 2003, Abdollahi et al. 2005)
	Resveratrol	N/A		(Aggarwal et al. 2004)
	Vitaxin	MedImmune Inc.		(Mikecz 2000)
	SB273005	SmithKline Beecham Pharmaceuticals	Rheumatoid arthritis	(Badger et al. 2001)
	SC55631	N/A	Osteoporosis	(Engleman et al. 1997)
$\alpha IIb\beta 3$	Chimeric 7E3 Fab (Abciximab/ReoPro®)	Centocor	Unstable angina, restenosis, stroke,	(Bennett 2001)
	Lotrafiban	SmithKline Beecham Pharmaceuticals	acute coronary artery disease	(Liu et al. 2000)

medullablastoma cells (Taga et al. 2002). It is applied in phase I and II to treat non-small lung cancer, prostate cancer, glioblastoma, pancreatic cancer, melanoma, and lymphoma (www.cancer.gov/clinicaltrials).

Small molecule integrin antagonists

There is evidence that small molecule antagonists could be used to treat human diseases, which depend on angiogenesis, including rheumatoid arthritis, osteoporosis, and cancer (Hartman & Duggan 2000, Kerr et al. 2000, Giavazzi & Nicoletti 2002, Shimaoka & Springer 2003). For this purpose, new classes of small molecule $\alpha v\beta 3$ antagonists have been developed, including isoxazolines (Pitts et al. 2000), disubstituted indazoles (Batt et al. 2000), and non-peptide chemical RGD peptidomimetics. An example of this latter class of antagonists, S247, which blocks $\alpha v\beta 3/\alpha v\beta 5$, was shown to decrease tumor growth and angiogenesis of colon cancer liver metastases leading to prolonged survival in an orthotopic murine model (Reinmuth et al. 2003). Another non-peptide chemical RGD mimetic that targets $\alpha v\beta 3$, SC56631 inhibits bone resorption in vitro and suppresses osteoporosis in oestrogen-deprived animals (Engleman et al. 1997). The naturally occurring polyphenol, Stilbene Resveratrol also binds to integrin $\alpha v\beta 3$ at or near the RGD-binding site and induces apoptosis in cancer cells through activation of ERK with consequent phosphorylation of p53 (Lin et al. 2006). Compared to $\alpha v\beta 3$, few small molecule antagonists are known to inhibit $\alpha 5\beta 1$. Nevertheless, treatment with ATN-161, a non-RGD peptide specific for $\alpha 5\beta 1$ that is derived from the synergy site in fibronectin that is part of the $\alpha 5\beta 1$-binding motif but not of that of $\alpha v\beta 3$, enhanced the efficacy of chemotherapy in a mouse model for colon cancer metastasis (Stoeltzing et al. 2003).

Monoclonal antibodies

LM609 is an anti-human integrin $\alpha v\beta 3$ mAb that blocks cell adhesion to RGD-containing ligands (Cheresh 1987). It prevents bFGF (basic fibroblast growth factor)- and TNFα (tumor necrosis factor-α)-dependent angiogenesis but has no effect on pre-existing vessels (Brooks et al. 1994a). LM609 was effective in preclinical models for glioblastoma, melanoma, breast, and prostate cancer. Intravenous administration of LM609 inhibited outgrowth of $\alpha v\beta 3$-negative human breast cancer cells in a combined (SCID) mouse/human chimeric model (Brooks et al. 1995). Fewer human blood vessels and less invasive tumors were observed in these LM609-treated animals with no apparent effect on normal human tissue, indicating that LM609 acts as an anti-angiogenic compound. Similar effects have been obtained with antibodies directed against $\alpha 5\beta 1$ (Kim et al. 2000).

The serum half-life and integrin-binding affinity of LM609 have been improved by generating a humanized version, Vitaxin (Carter 2001, Wu & Senter 2005). Optimization of the complementarity-determining regions further improved Vitaxin's integrin binding affinity allowing it to inhibit tumor growth in Kaposi's sarcoma and partially inhibit the binding of the human immunodeficiency virus-1 (HIV-1) Tat protein to $\alpha v\beta 3$ (Rader et al. 2002). Vitaxin II, although binding $\alpha v\beta 3$ with even higher affinity, showed no positive response in cancer treatment but may be effective as adjuvant in combination with chemo- or radiation therapy (Posey et al. 2001). In 2003, MedImmune licensed Vitaxin II (MEDI-522) for clinical development in phase II trials in prostate cancer, melanoma, psoriasis, and rheumatoid arthritis. However in 2004, the clinical trial of Vitaxin in the treatment of rheumatoid arthritis and psoriasis was ended because preliminary results failed to demonstrate clinical benefits. Nevertheless, the trials for melanoma and prostate cancer are still in progress (MedImmune ends some Vitaxin testing, advanced tests for arthritis treatment halted; cancer research continues. Article by Michael S. Rosenwald, *Washington Post* staff writer; August 31, 2004; Page E05).

Strongly improved versions of antibodies against $\alpha 5\beta 1$ have also been generated. For instance,Volociximab, a chimeric humanized mAb, is a high affinity function inhibitor of the $\alpha 5\beta 1$ integrin, which, like $\alpha v\beta 3$ has been found to be upregulated in activated endothelial cells. It has been applied in clinical phase II trials for solid tumors in renal cell carcinoma, metastatic melanoma and pancreatic cancer. These are ongoing studies with no data reported yet (www.clinicaltrials.gov).

Potential of integrin antagonists to improve efficacy of existing anticancer therapy

Similar to other anti-angiogenic agents, integrin antagonists can be applied in combination with cytotoxic anticancer therapy, such as chemo- or radiotherapy. Such combinational approaches can maximize efficacy in cancer by destroying cancer cells as well as endothelial cells, the latter depriving the tumor of nutrients and oxygen (Teicher 1996). On the other hand, the anti-angiogenic agent may also 'normalize' the abnormal structure and function of tumor vessels, thereby improving drug (but also oxygen) delivery (Jain 2005). Examples of such combination therapies are discussed in the following (Figure 2).

The ATN-161 $\alpha 5\beta 1$ antagonist enhanced the efficacy of chemotherapy in a mouse model for colon cancer metastasis (Stoeltzing et al. 2003). Co-application with Cilengitide increased the anti-tumor effectiveness of a tumor-specific antibody against interleukin 2 (IL-2) fusion proteins in a murine tumor model (melanoma, colon carcinoma, and neuroblastoma) (Lode et al. 1999). In pancreatic cancer, Cilengitide combined with gemcitabine (a radiation-sensitizing agent and a wide spectrum anti-cancer drug) inhibited highly vascularized tumor growth (Colomer 2004, Raguse et al. 2004). In ongoing trials for breast cancer, colon cancer, prostate cancer, melanoma, lung cancer, glioblastoma, and ovarian cancer, Vitaxin II is applied in combination with chemo-, hormonal-, biological-, immuno-, or radiotherapy. Patients with Stage IV

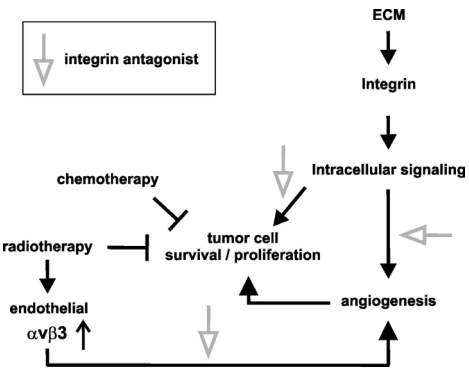

Figure 2. Integrins as targets in anti-cancer therapy. Open arrows indicate processes that are blocked by integrin antagonists. These include survival and proliferation of tumor cells as well as survival, proliferation, and/or migration of endothelial cells. The latter may be of particular importance during radiotherapy where increased expression of $\alpha v\beta 3$ on endothelial cells can mediate therapy escape through enhanced angiogenesis.

melanoma have 9.4-month median survival when treated with Vitaxin II combined with dacarbazine (DTIC) whereas patients treated with DTIC alone, have a median survival of 7.9 months (Cai & Chen 2006). It has been reported that radiotherapy in fact promotes integrin-mediated survival signaling through PKB/Akt by upregulating $\alpha v\beta 3$ expression on endothelial cells. This escape mechanism can be circumvented by administering angiogenesis inhibitors, such as the small molecule $\alpha v\beta 3$ integrin antagonist S247, which prevents radiation-induced PKB/Akt phosphorylation leading to enhanced anti-angiogenic and anti-tumor effects (Abdollahi et al. 2005).

Most integrin antagonists interfere with binding of natural ECM components to their receptors and thereby prevent integrin signaling to survival and proliferation. As such, integrin antagonists may effectively suppress tumorigenesis by targeting these signaling pathways in both tumor and endothelial cells. Ultimately, a trimodal strategy in which radio-, chemo-, and anti-angiogenic therapies are combined may be highly effective in the treatment of cancer. The use of integrin antagonists as anti-angiogenic agents that may also target tumor cells, may fit well in such strategies.

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Chapter 6

Automated microinjection of cell-polymer suspensions for high throughput quantitiative cancer invasion screens.

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Automated microinjection of cell-polymer suspensions for high throughput quantitiative cancer invasion screens.

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Cell spheroids (CS) embedded in 3D extracellular matrix (ECM) serve as in vitro mimics for multicellular structures in vivo. Such cultures, started either from spontaneous cell aggregates or single cells dispersed in a gel are time consuming, applicable to restricted cell types only, prone to high variation, and do not allow CS formation with defined spatial distribution required for high-throughput imaging. Here, we describe a novel method where cell-polymer suspensions are microinjected as droplets into collagen gels and CS formation occurs within hours for a broad range of cell types. We have automated this method to produce CS arrays in fixed patterns with defined x-y-z spatial coordinates in 96 well plates and applied automated imaging and image analysis algorithms. Low intra- and inter-well variation of initial CS size and CS expansion indicates excellent reproducibility. Distinct cell migration patterns, including cohesive strand-like - and individual cell migration can be visualized and manipulated. A proof-of-principle chemical screen is performed identifying compounds that affect cancer cell invasion/migration. Finally, we demonstrate applicability to freshly isolated mouse breast and human sarcoma biopsy material - indicating potential for development of personalized cancer treatment strategies.

INTRODUCTION

Cells grown under classical 2D culture conditions behave differently from the same cell types grown *in vivo*. In addition to soluble factors produced in the *in vivo* microenvironment, differences in cell shape, intercellular contacts, and connections to ECM have striking effects on gene expression, cell survival, proliferation, differentiation, cytoarchitecture, and migration. Various systems have been developed to culture cells within 3D ECM environments, aimed at more closely mimicking the *in vivo* context[1,2]. Several of these systems produce 3D cell aggregates in which, after compaction, depletion of oxygen, nutrients, and growth factors occurs in the core, leading to cell heterogeneity depending on the position in the resulting

cell spheroids (CS)[3,4]. Multistep methods are used in which aggregates are allowed to form spontaneously and, following a compaction phase, can subsequently be transferred to a 3D ECM. The best-known example of this approach is the “hanging drop assay” that was developed to create embryoid bodies from ES cells and has also been applied to cancer cell lines to produce tumor-like structures[5,6]. Alternative methods involve mixing of single cell suspensions with a solidifying ECM, resulting in individual cells that eventually form CS randomly within a 3D ECM structure[7], or seeding polymeric scaffolds with cell/ECM suspensions[2].

Cell behavior in 3D cultures is controlled by chemical (composition) and physical (rigidity, cross-linking) properties of the gel. Natural ECM proteins can be used such as collagen, fibrinogen, or the laminin-rich matrigel to represent the *in vivo* ECM composition most relevant to a given cell type. More recently, synthetic polymers have been developed for 3D CS culture environments although it remains to be established how well these support a variety of cell behavioral outputs, including cell migration[8]. Collagen type 1 is an abundant polymer in ECM *in vivo*, and it is widely used for 3D cultures. Various physical properties of the collagen gel, such as rigidity and pore size modulate stem cell differentiation, cancer growth, and cell migration[9-11]. Cells can use various migration strategies in 3D environments, including mesenchymal or amoeboid individual cell migration modes or collective invasion strategies, depending on properties of the cells and of the matrix[10]. Changes in matrix pore size can force cells to adopt alternative migration strategies or - if too extreme - pose a barrier to cell migration. Importantly, cells can modify the ECM by physical deformation and proteolysis, to overcome such barriers[12].

Chemical compound screens as well as RNAi screens for various types of cellular functions, including survival, growth, differentiation, and migration are mostly performed in 2D culture conditions. Methods to analyze cells in 3D based on the hanging-drop assay are labor and time intensive; are limited to cell types that are cohesive and aggregate spontaneously; and are prone to high variability between experiments due to variation in aggregation and compaction time and CS size. Alternative methods in which single cell suspensions are mixed with soluble ECM substrates that are subsequently allowed to form a gel are relatively easy to perform but also have several major disadvantages: formation of CS depends on the ability of a cell type to survive and proliferate as single cells in low adhesion conditions for extended periods; CS formation is time consuming; CS show a large variation in size; and CS form at random locations, which is disadvantageous for imaging purposes.

To allow for CS formation that is relatively fast and easy, highly reproducible, and overcomes the disadvantages described above we have developed a novel method where cell-polymer suspensions are microinjected into multiwell plates containing a collagen gel. This method has been automated to produce CS arrays with highly reproducible properties in large quantities in 96 well plates. We use this system to visualize distinct 3D migration strategies and regulation of those strategies by ECM properties and actomyosin contractility. We demonstrate applicability in high-throughput screening platforms in a chemical screen for compounds that affect breast cancer invasion/migration. Finally, we apply the method to cell suspensions derived from fresh tumor biopsies, which opens the possibility to test

therapeutic strategies on freshly isolated material from individual patients.

MATERIALS AND METHODS

Cell culture

The following cell lines were obtained from ATCC: MDA-MB-231, MTLn3, PC-3, HT1080, 4T1, and MAE. GEβ1 was described earlier[13]. All cell lines were cultured under standard cell culture conditions indicated by ATCC or as described[13] at 37ºC, 5% CO_2 in a humidified incubator. Primary mouse tumor cell suspensions were derived from surplus mouse breast tumor material by mincing using scalpel and tissue chopper followed by 2-hour collagenase treatment at 37°C. Human biopsy material was obtained from surplus material from patients that were surgically treated for chondrosarcoma or osteosarcoma. Tumor cell suspensions were derived from biopsies by 12h collagenase treatment at 37ºC. All human specimens were handled in an anonymized coded fashion according to the National ethical guidelines for secondary use of patient-derived material.

Preparation of collagen

Collagen type I solution was obtained from Upstate-Milipore or isolated from rat-tail collagen by acid extraction as described previously[14]. Collagen was diluted to indicated working concentrations of ~2.4 mg/ml in PBS containing 1xDMEM (stock 10x, Gibco), 44 mM $NaHCO_3$ (stock 440 mM, Merck), 0.1 M Hepes (stock 1M, BioSolve).

Hanging drop method

~5,000 cells in 20 μl droplets were dispensed onto a 10 cm dish that was inverted over a dish containing 10 ml DMEM. After 24h, cell aggregates were harvested using a Pasteur pipette and transferred into 10 cm dishes coated with 0.75% agarose submerged in 10ml DMEM. After 48h CS had formed and these were embedded into a 2.4 mg/ml collagen solution using a Pasteur pipette. Collagen gels were allowed to solidify at 37ºC for 30 min and overlaid with DMEM. Cell invasion was recorded for 3 days using an inverted phase contrast light microscope (Nikon Eclipse E600).

Cell preparation for injection method

Cell suspensions derived from trypsin-detached adherent cultures or from collagenase-treated biopsies were filtered to remove clumps, centrifuged at 1000 rpm for 5 minutes, and washed twice with PBS. ~$7x10^6$ cells were re-suspended in 30 μl PBS containing 2% polyvinylpyrrolidone (PVP; Sigma-Aldrich). The PVP/cell suspension was loaded into a beveled pulled glass needle (Eppendorf CustomTip Type III, oD [μm] 60, Front surface 40, Flexibility: rigid).

Manual injection

Cell suspensions in 2% PVP were microinjected (~$1x10^4$ cells/droplet) with a microinjector (20 psi, PV820 Pneumatic PicoPump, World Precision Instruments, Inc) into solidified collagen gels in 8 well μslides (IBIDI).

Automated injections

A glass-bottom 96 well plate (Greiner) containing 60μl solidified 2.4 mg/ml collagen gel per well was placed in a motorized stage (MTmot 200x100 MR, Märzhäuser) connected to a controller (Tango, Märzhäuser). A motorized micro-manipulator (Injectman II, Eppendorf) was positioned above the stage and connected to a pump (Femtojet Express, Eppendorf) featuring an external compressor (lubricated compressor, model 3-4, JUN-AIR). A firewire camera (DFK41BF02.H, The Imaging Source) equipped with an 8x macro lens (MR8/O, The Imaging Source) was placed beneath the stage for calibration and imaging. All components were connected to the controlling computer (Ubuntu AMD64). A multi-threaded control program was written in Python using PySerial and wxPython. Coriander software (http://damien.douxchamps.net/ieee1394/coriander) was used for imaging.

After the program was calibrated for the 96 well plate the camera height was adjusted to focus on the bottom of the 96 well plate. The plate was then removed for needle calibration: the injection needle was fixed in the Injectman and moved, using the Injectman controller, into the center of the image. The injection height was set to 200μm above the bottom of the (virtual) plate. After the needle was moved up, the plate was placed back in position and the upper left well was used for multiple test injections to adjust pump pressure and injection time for optimization of the droplet size (~8nl ≈ 300μm diameter) using video inspection. Subsequently, using a pre-defined macro defining x-y coordinates and number of injections per well, all wells were injected with the same pressure and injection time.

Microscopy and image analysis

Manually injected CS were monitored daily using a Nikon Eclipse E600 microscope. CS generated by automated injection were used for montage imaging using a Nikon TE2000 confocal microscope equipped with a Prior stage controlled by NIS Element Software and a temperature and CO_2-controlled incubator.

Differential interference contrast (DIC) images were captured using a charged coupled device (CCD) camera with NIS software at 10x dry objective. Quantification of CS invasion area was analyzed from DIC images using ImageJ. The CS ellipsoidal area after three days was estimated using the diameter in x and y axis (pi*radius-x*radius-y) occupied by cells in the 10x montage image in the mid-plane of each CS and normalizing to the occupied area 1h after injection. One-way ANOVA was performed to test the significance of the data. The data are presented and plotted as average and standard error of the mean.

For automated imaging, wells containing gel-embedded CS were treated with a fixation and staining cocktail containing 3.7% paraformaldehyde, 0.2% Triton X-100 (Sigma) and 0.1 μM rhodamine Phalloidin (Sigma) for 3 hrs. Wells were washed extensively with PBS and plates were imaged on a Becton Dickinson Pathway 855 using a 4X lens. A montage of 12 frames was made for each Z plane, with a total of 24 Z planes at an interval of 50 μm. Image stacks were converted into 2D maximum fluorescence intensity projections using ImagePro 7.0. CS were then digitally segmented using ImageJ to identify the outline of individual CS and multiple parameters were measured, including Feret's diameter, roundness, and number of CS scored in each well.

For immunostaining of E-cadherin, gels were incubated for 30 mins with 5 μg/ml collagenase (Clostridium histolyticum, Boehringer Mannheim) at room temperature, fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 10% FBS.

Gels were incubated with E-cadherin antibody (BD Transduction Laboratories) overnight at 4°C followed by Alexa 488-conjugated secondary antibody (Molecular Probes/Invitrogen) for 2 hrs at room temperature and Hoechst 33258 nuclear staining (Molecular Probes/Invitrogen) for 30 min at room temperature. Preparations were mounted in Aqua-Poly/Mount solution (Polysciences, Inc) and analyzed using a Nikon TE2000 confocal microscope. Z-stacks (~100 stacks, step of 1 μm) were obtained using a 20x dry objective, imported into ImageJ, and collapsed using extended depth of field plugin (Z projection) into a focused composite image.

Drug Treatment

LY-294002 (phosphatidylinositol 3-kinase), JSI-124-cucurbitacin (STAT3/Jak2), NSC23766 (Rac1), and AG-82 (general protein tyrosine kinases) were purchased from Merck/CalBiochem. PP2 (Src) and ML-7 (MLC kinase) were purchased from ENZO. Y-27632 (Rock), SB-431542 (TGFβ) and AG1478 (EGFR) were purchased from BioMol Tocris. Cell migration was analyzed in the absence and presence of inhibitors for 4 days.

RESULTS

Development and characterization of the method

To design a protocol that rapidly produces CS with highly reproducible characteristics we developed a novel method based on microinjection. For the microinjection method we mixed cells with polyvinylpyrrolidone (PVP), which is an inert (hydrophilic) water-soluble synthetic polymer, also used as emulsifier, food-additive (E1201) and as solubilizing agent for injections[15]. In our application it was used to delay cell sedimentation within the capillary needle. Furthermore, in our experience cells rapidly disperse in the absence of PVP while cells injected in the presence of PVP remained localized (e.g. trapped by the polymer) at the site of injection, allowing time for aggregation and CS formation.

We first compared our method to the established hanging drop assay[5]. 20 μl drops containing $5x10^3$ GEβ1 cells were used to create hanging drops in an inverted 10 cm dish (**Fig 1a**). The time required to form cell aggregates was 24h. These cell aggregates were transferred to agarose-coated dishes where they formed tightly packed CS over a period of 48h. Next, the CS were embedded in 2.4 mg/ml collagen solution that was subsequently allowed to solidify. For microinjection, GEβ1 cells were suspended in 2% PVP, loaded into a pulled glass needle, and ~80nl droplets containing ~$1x10^4$ cells were injected directly into preformed 2.4 mg/ml collagen gels where they formed tightly packed CS within 1h (**Fig 1a**). Microinjection-derived CS at 24h post-injection and hanging-drop-derived CS at 96h post initiation (24h in collagen) displayed similar cell migration the following days (**Fig 1b**). Microinjection-derived CS were also established from 4T1 mouse breast carcinoma cells where E-cadherin staining marked cell-cell contacts within the first day post-injection that were maintained for at least 96h (**Fig 1c**).

CS derived by microinjection of different cancer - or non-cancerous cell types allowed analysis of various distinct motile strategies in 3D (**Fig 1d**). Cell types that do not typically form cell-cell contacts in 2D cell culture (and that are typically difficult to study in 3D using the hanging-drop-, liquid overlay-, or other assays in absence of additives like matrigel[16,17]) such as MTLn3 and MDA-MB-231 breast cancer cells and HT1080 fibrosarcoma cells, displayed amoeboid (MTLn3) or mesenchymal (HT1080 and MDA-MB-231) movement of individual

cells. On the other hand, 4T1 breast cancer, PC-3 prostate cancer, and human microvascular endothelial cells (HMEC) that grow as islands in 2D culture, invaded as cohesive strands into nto collagen matrix.

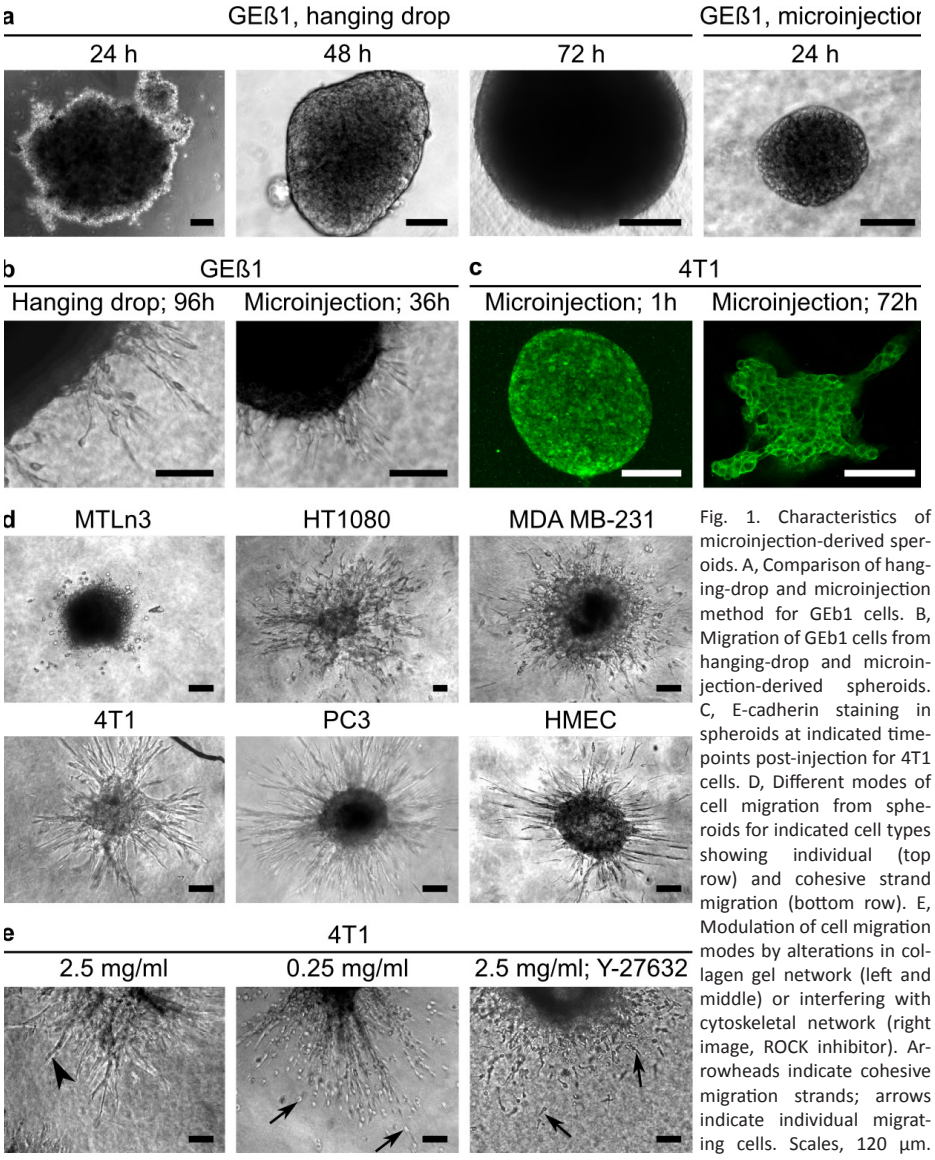

Fig. 1. Characteristics of microinjection-derived sperоids. A, Comparison of hanging-drop and microinjection method for GEb1 cells. B, Migration of GEb1 cells from hanging-drop and microinjection-derived spheroids. C, E-cadherin staining in spheroids at indicated timepoints post-injection for 4T1 cells. D, Different modes of cell migration from spheroids for indicated cell types showing individual (top row) and cohesive strand migration (bottom row). E, Modulation of cell migration modes by alterations in collagen gel network (left and middle) or interfering with cytoskeletal network (right image, ROCK inhibitor). Arrowheads indicate cohesive migration strands; arrows indicate individual migrating cells. Scales, 120 µm.

ECM rigidity influences cell behavior in 3D and the actin cytoskeleton is believed to be essential for sensing and responding to such physical ECM properties[12]. We used these CS to study the effect of alterations in ECM network composition or intracellular cytoskeletal network properties on migration strategies in 3D. Lowering ECM rigidity by decreasing collagen concentration from 2.5 to 0.25 mg/ml or lowering cytoskeletal tension by application of a ROCK inhibitor, both caused a switch from cohesive strand invasion to individual cell migration in 4T1 cells (**Fig 1e**). This suggests that tension exerted on cell-cell adhesion structures either from outside or inside the cell is required for cohesive 3D movement.

Altogether, these results demonstrate that the microinjection method produces CS for 3D growth and migration studies rapidly (hours versus days), conveniently (one step), with a broad spectrum of cell types including those that are incompatible with previous methods, and displaying a variety of migration patterns.

Method automation

Since this novel method has the potential to rapidly create CS with high reproducibility for large-scale analysis in 3D ECM of cell growth and migration/invasion we set up a procedure to automate the CS formation process. For this purpose, a 96 well plate containing 60 μl collagen gel per well was placed on a motorized stage and the glass needle containing the cell/PVP suspension described above was placed vertically in a motorized micromanipulator above the stage (**Fig 2a**). After calibration of needle and 96-well plate using camera vision from under the stage, a computer script was used to automate the injection process with various macros.

With this set up, cell droplets were injected resulting in CS of ~300 μm diameter (**Movie S1**). To increase reproducibility, using commercial needles reduced needle tip diameter variance and gels were prepared from a single large batch of collagen isolated in-house from rat-tail. Various layouts of injection patterns were tested. A hexagonal pattern of 19 CS spaced at 1.2 mm started to show interaction between migration strands of CS at day 4 but a less dense hexagon pattern of 7 CS at 2 mm spacing provided sufficient spacing for 96h analysis of CS migration (**Fig 2b,c**). Visual inspection indicated reduced CS migration on the most outer rows and columns of each plate, pointing to edge effects. We therefore chose to exclude these wells in all further experiments. We determined reproducibility in all other wells and detected no significant intra- or inter-well variation in initial CS size (ANOVA, $P>0.5$) or CS expansion over ~92h (ANOVA, $P>0.5$) (**Fig 2d**).

These data demonstrate that the microinjection method can be automated to create with high reproducibility and predefined x-y-z coordinates CS arrays in 96 well plates. Such properties make this protocol ideal for automated imaging strategies.

Application of the method to automated drug screens

A proof of principle drug screen was performed to test the applicability of this procedure to automated high-throughput drug screening assays (HTS). 4T1 CS were generated and various previously described inhibitors, including AG1478 (EGFR), PP2 (Src), ML-7 (MLCK), Y-27632 (ROCK), NSC23766 (Rac), SB-431542 (TGFβR activin-like kinases), AG-82 (EGFR), LY-294002 (PI3K), JSI-124 (STAT3) were added one hour later at different concentrations (4, 10, 20 μM) in duplicate. Effects on cell migration could be clearly observed by DIC imaging after 2 and 4 days for ML-7 and JSI-124 (**Fig 3a**). For automated imaging and image analysis protocols, we labeled the actin cytoskeleton at day 4 of all 10 μM-treatments and controls (**Fig 3b**). This allowed automated capture of Z-stacks that were converted to maximum projection images, thresholded, and used for automated multiparameter analysis including Feret's diameter and circularity.

Visual inspection and manual assessment of Feret's diameter from DIC images at day 0 and 4 demonstrated that initial CS size, CS expansion, and inhibition of invasion by ML-7 and JSI-124 were highly reproducible (**Fig 3a,c**). Automated image analysis fitted well with these data showing that ML-7 and JSI-124 caused significantly reduced Feret's diameters ($p<0.05$) (**Fig 3d**). For JSI-124 this correlated with increased circularity ($p<0.05$) in agreemen

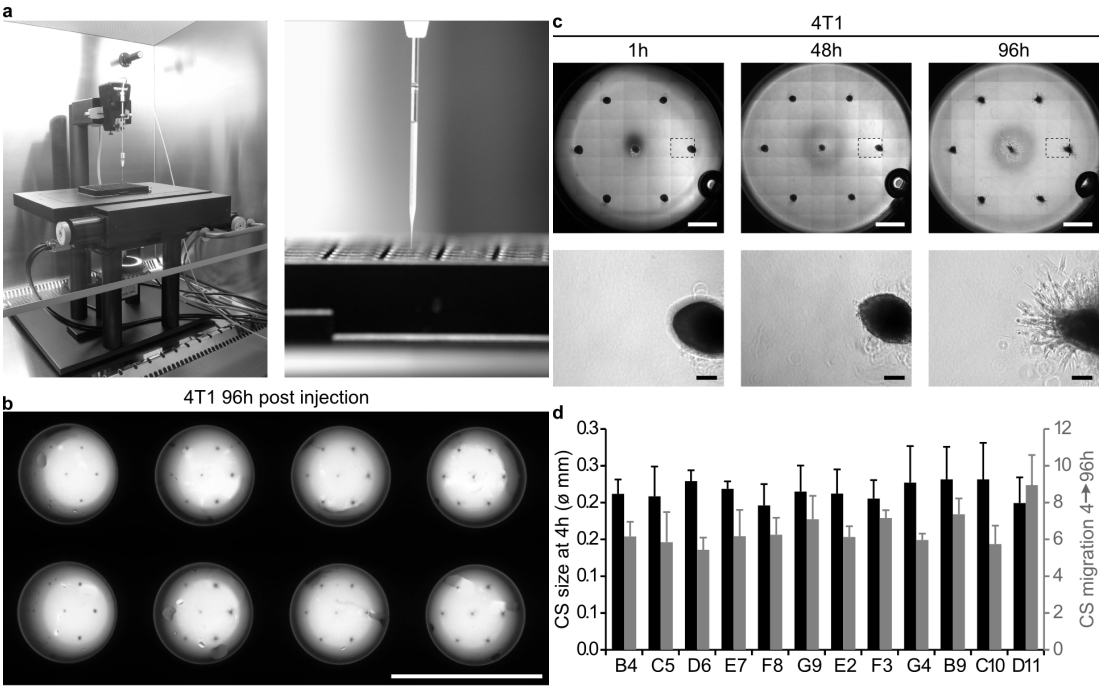

Fig. 2. Automated production of spheroid arrays. A, Automated injection system (left) and cell/PVP suspension in needle during injection (right). B, Bottom view of multiple wells with 4T1 spheroid arays 96 hours post-injection. Scale, 10mm. C, Upper row shows stitched brightfield images showing spheroid arrays at indicated timepoints. Scale, 1 mm. Bottom row shows cell migration from single bright-field images of spheroids marked by dashed rectangle in upper row. Scale, 100 µm. D, Mean and SD for initial spheroid size 4h post-injection (black bars) and CS migration over ~4 days determined from outline of migration strands (grey bars) obtained from all 7 spheroids /well for indicated wells of a 96-well plate.

with inhibition of invasion and a remaining round CS. The extremely low values observed for ML-7 (**Fig 3d**) despite the fact that a CS was observed by DIC (**Fig 3a**) can be explained by ML-7-induced loss of filamentous actin fibers causing reduced staining in this particular method. Alternative staining procedures should lead to improvement and compatibility with real-time analysis. Nevertheless, the reproducibility of the injection procedure (**Fig 2 and 3**) combined

with the similarity between visual inspection and automated imaging (**Fig 3c,d**), demonstrates that this automated injection system can be coupled to fully automated imaging and image analysis methodology that is accurate and reproducible.

Compatibility of the method with primary biopsy material

We determined if this methodology is compatible with freshly isolated biopsy material. First, a cell suspension was generated from 4T1-GFP orthotopic breast tumors in mice using collagenase-treatment. In contrast to alternative methods, the microinjection method circumvents any 2D tissue culture steps, which may cause altered cell behavior[18-22]. Following injection, these cells rapidly formed CS from which migration was analyzed after 3 days (**Fig 4a**). CS were stained for actin and Hoechst and the near complete overlap between actin and GFP staining demonstrates that these CS consist mainly of tumor cells.

Next, cell suspensions were derived by collagenase treatment of freshly isolated human osteosarcoma and chondrosarcoma tissue. Following injection, CS readily formed from these human biopsies and survival and migration could be studied for up to one week with the two tumor types showing distinct migratory behavior (**Fig 4b**). Osteosarcoma

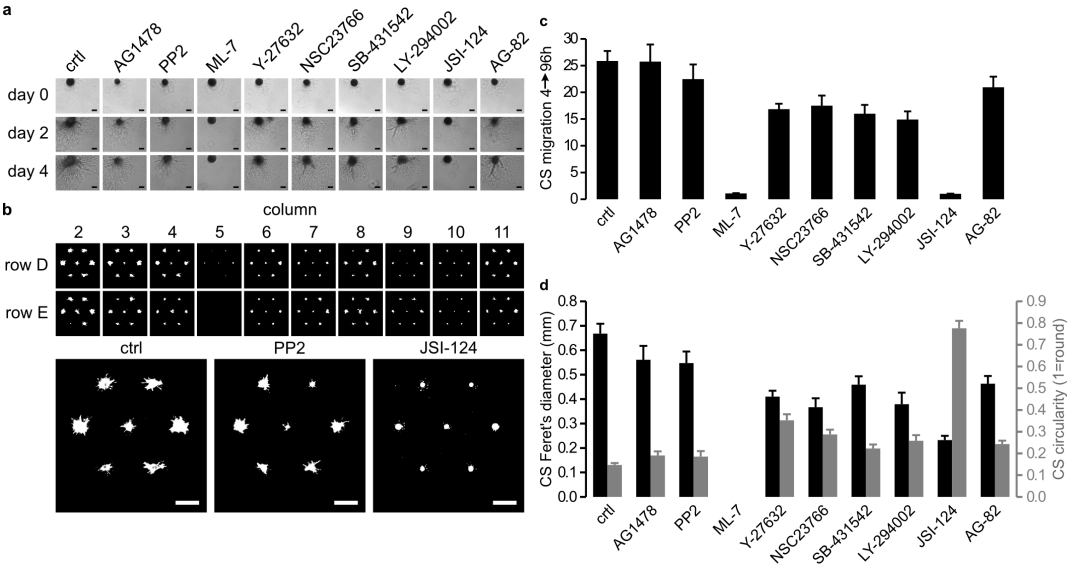

Fig. 3. Results from a drug screen performed on 4T1 cells in a 96 well plate. A, DIC images showing tumor cell migration in the presence of indicated inhibitors at indicated timepoints (scale = 100 µm). B, Top 2 rows, rhodamine-phalloidin staining and thresholding for indicated wells at 4 dpi (columns correspond to treatments from A; rows represent duplicates); bottom row, zoom in on well D2 (Ctr), D4 (PP2), and D10 (JSI-124). Scale, 1 mm. C, effect of indicated inhibitors on CS migration over ~4 days determined from outline of migration strands derived from DIC images in A (mean and SD for 14 spheroids derived from 2 wells is shown). D, Quantification of data derived from automated analysis of fluorescent images shown in B (mean and SD for 14 spheroids derived from 2 wells is shown).

mainly displayed individual amoeboid movement whereas chondrosarcoma showed predominantly individual mesenchymal movement. We treated these CS with the range of compounds described above at 10 µM starting 1 day post-injection. Several of the chemical inhibitors effectively inhibited migration of both tumor types (**Fig 4b,c**). Notably, the ROCK inhibitor Y-27632 did not affect mesenchymal movement but caused switching from amoeboid to mesenchymal movement in the osteosarcoma cells, in line with the described requirement for ROCK activity only in amoeboid single cell movement[23].

Taken together, these data indicate that the automated CS injection methodology has the potential to be used for drug testing on tumor cells freshly isolated from individual patients.

DISCUSSION

Here, we describe a method for generation of 3D CS cultures based on microinjection of cell suspensions into premade gels, that has a number of features making it highly useful for drug screening applications: compared to previous methods it is easy (one step procedure) and fast (minutes instead of days); CS are generated with high accuracy at predetermined x-y-z positions in multiwell plates; it is applicable to many different cell types irrespective of the ability of cells to form spontaneous cell-cell contacts; it shows good intra- and inter-well reproducibility with respect to CS size and migration; because of the predefined coordinates of each individual CS the method can easily be combined with fully automated imaging and image analysis protocols (**Fig 4d**).

2D culture conditions are a very poor representation of the environment cells encounter *in vivo*. Besides implications for cell biology studies, this has important consequences for the interpretation of genetic – and drug screens[24]. So far, these

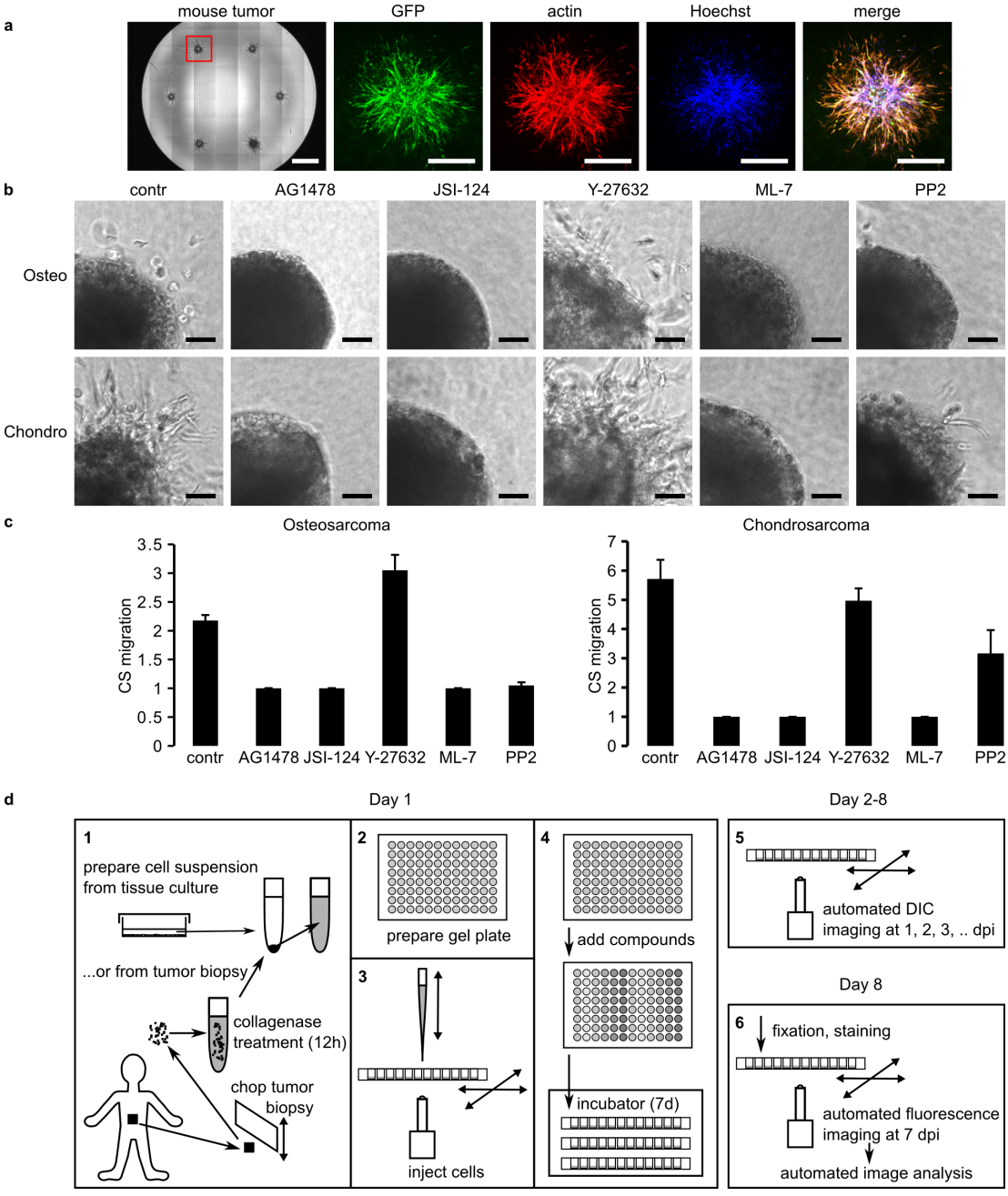

Fig. 4. Application to tumor biopsies. A, Overview DIC image (left) and zoom in on individual spheroid obtained from 4T1-GFP orthotopic mose breast tumor. Scales, 1 mm (left DIC); 500µm (fluorescent images). B, DIC images showing spheroids derived from osteosarcoma (top) and chondrosarcoma biopsy (bottom) treated with indicated inhibitors. Scale, 100µm. C, effect of indicated inhibitors on CS migration over ~4 days determined from outline of migration strands derived for DIC images in A (mean and SD 12 spheroids derived from 2 wells is shown). D, Schematic overview of high-throughput spheroid screening indicating procedure at day 1 (steps 1-3) and imaging in absence or presence of compounds at days 2-8 (steps 4-6).

have mostly been performed on 2D cultures. For the study of tumor cell invasion the Boyden chamber assay (trans-well migration assay) is also commonly used. Here, a monolayer of cells migrates through a thin layer of gel to reach the bottom of a filter. This particular assay

does not resemble cells disassociating from a solid tumor. For this purpose, CS cultures have been developed that provide a pathophysiological context that mimics solid cancer microenvironments. However, these have not been used for large-scale drug screens due to the complicated procedures, which negatively affect reproducibility of results and lead to higher costs. Reproducibility of CS size is critical for a reliable 3D culture platform. Size and compactness of CS will inevitably affect drug penetration and previous studies have indicated that CS with diameters between 200 and 500µm are required to develop chemical gradients (e.g. of oxygen, nutrients,and catabolites) that may represent conditions found in tumors[3,4,25-28]. Our automated approach yields CS with a diameter of ~300µm, a size that may thus represents solid tumor traits.

We have used collagen-based gels but the same method could be easily adapted to studies using alternative 3D matrices. The type and concentration of matrix proteins will have considerable influence on scaffold structure, rigidity, and porosity, which will impact on cell morphology, survival, proliferation, and migration efficiency[8,29,30]. We find that changing collagen concentrations has a major impact on CS cell migration and that optimal conditions differ for distinct cell types, in agreement with findings from others[8,29-32]. Hence, it is essential that gel formation is standardized and optimized for each cell type. The use of ECM proteins such as collagen has some limitation in terms of controlling batch-to-batch variation. Therefore, chemical crosslinking stabilization may be applied to better control the mechanical properties (porosity and mechanical strength). A number of different cross-linking agents that react with specific amino acid residues on the collagen molecule, synthetic biopolymer scaffolds, and self-assembling synthetic oligopeptide gels are available to address this[29,30,33,34].

We demonstrate that we can automate each step of the procedure, from injection of cell suspensions to imaging and image analysis, while maintaining reproducibility. Our method not only accelerates and simplifies CS formation but by generating up to 7 CS per well at predefined x-y-z coordinates it is compatible with fully automated imaging procedures, enhanced data collection, and robust statistical analysis. We present a small drug screen to demonstrate such properties. Finally, we show that the method presented here can be used for CS formation directly from freshly isolated tumor biopsy material without the need of any intermediate culture steps. This eliminates artificial traits induced by 2D culture. A fully analyzed CS cancer migration screen in 96 well plates can be derived from a biopsy within 1 week. This opens the door to screening on a patient-by-patient basis for drug sensitivity of tumor cells under conditions that may closely mimic the *in vivo* pathophysiological situation. Clinical tests to validate inhibitor effects in CS screens by comparing with therapeutic efficacy can be performed without further modifications of the presented system. Moreover, expansions of this method can be envisioned in which multiple cell types are combined (e.g. cancer cells and cancer-associated fibroblasts and/or endothelial cells) to further improve representation of the complex tumor microenvironment.

CONCLUSIONS

Current methodologies do not allow formation of multicellular structures in 3D ECM scaffolds with defined spatial distribution at high speed and high-throughput, properties that are especially important for use in high content screening (HCS) platforms. In our novel method, cell-polymer suspensions are microinjected as droplets into solidified collagen gels. CS formation time is strongly reduced compared to other methods (minutes rather than days) and it can be applied to a broad range of cell types. Several distinct 3D cell migration strategies are observed and can be manipulated. For HCS, the method has been automated to produce CS arrays with defined x-y-z spatial coordinates in multiwell plates and coupled to automated imaging and image analysis algorithms. Low intra- and inter-well variation allows chemical screening for compounds affecting cancer cell invasion. CS can be derived from primary mouse and human tumor biopsy material without intermediate culture steps to be subjected to image-based 3D compound screening.

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AUTHOR CONTRIBUTIONS

HHT and JdS and EHJD conceived experiments and wrote the manuscript. HHT, JdS, VPSG and JX performed experiments. LP and JdS performed automated image analysis. PH isolated tumor material. HS and BvdW contributed to technology developmen

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Mov. 1. Automated injection-derived spheroids. Left panel shows top view of needle moving over 96-well plate; right panel provides bottom view of injection of cell/PVP suspensions into collagen gels.

Chapter 7

Integrin control of ZEB/miR-200 balance regulates tumor cell migration strategy and metastasis

Truong HH, Ghotra VPS, Nirmala E, Le Dévédec SE, van der Helm D, Lalai R, He S, Ewa Snaar-Jagalska BE, Amiet A, Marcinkiewicz C, Vreugdenhil E, Meerman JHN, van de Water and Danen EHJ. *Submitted for publication*

Integrin control of ZEB/miR-200 balance regulates tumor cell migration strategy and metastasis

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Cellular interactions with the extracellular matrix (ECM) are mediated by transmembrane receptors of the integrin family, which coordinate signal transduction cascades impinging on cell survival, proliferation, and migration[1]. Interactions through β1 integrins support initiation and growth of breast and skin cancers and β1 antibodies sensitize breast tumors in mice to radiotherapy[2-4]. Here, we show that peptide blocking or gene silencing of β1 integrins can also cause a switch from cohesive multicellular strand invasion to individual cell migration in 3D matrices and in a zebrafish xenograft model where β1 integrin depletion promotes breast cancer cell spreading. In an orthotopic mouse transplantation model, tumor growth of β1 integrin-depleted breast cancer cells is reduced, but intravasation and lung metastasis of cells from these small tumors is strongly enhanced. Depletion of β1 integrins alters the balance between miR-200 microRNAs (miRNAs) and ZEB transcriptional repressors resulting in a transcriptional downregulation of E-cadherin, which is essential for the induction of individual cell migration and enhanced metastasis. These findings demonstrate that disturbed integrin-mediated interactions with the ECM in cancer cells can attenuate tumor growth but also alter cell migration strategies leading to increased metastasis through a miRNA-transcription factor network that controls cell-cell adhesion.

A panel of breast epithelial cell lines was microinjected into collagen gels where they formed tumor cell spheroids within the first day and cell migration was analyzed over the next 4 days. MTLn3 and MDA-MB-231 carcinoma cells, which lack E-cadherin-mediated cell-cell contacts in 2D culture, migrated into the collagen as single cells; a process here referred to as "*individual cell migration*" (not shown). MCF10a immortalized epithelial cells and 4T1 carcinoma cells, both forming E-cadherin-mediated intracellular junctions in 2D, invaded the collagen gel as multicellular strands; here referred to as "*cohesive invasion*" (Fig 1A, S1; Mov S1). We asked how integrin-mediated ECM attachment regulates cohesive invasion of cells with a relatively stable epithelial phenotype (MCF10A) and transformed cells displaying a mix of epithelial and mesenchymal characteristics (4T1). Silencing β1 integrins in MCF10a blocked invasive capacity whereas 4T1shβ1 spheroids lost the ability to invade as cohesive strands but instead

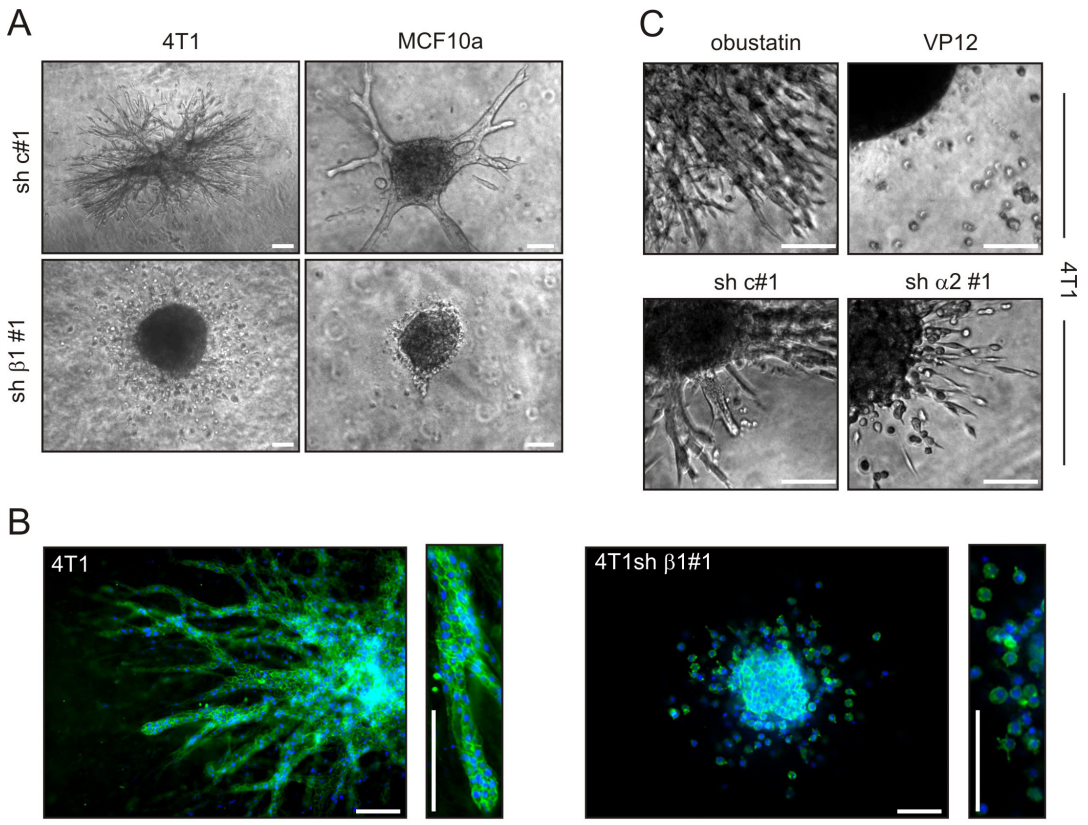

Figure 1. Integrin control of 3D migration patterns. A, spheroids of 4T1 and MCF10a cells expressing indicated shRNAs 4 days post-injection in collagen gels. **B,** E-cadherin staining of 4T1 and 4T1shβ1 spheroids 4 days post-injection in collagen gels. **C**, spheroids of 4T1 cells in the presence of indicated peptides (top) or expressing indicated shRNAs (bottom) 4 days post-injection in collagen gels. Scale bars, 50 μm.

tumor cells was strongly increased for 4T1shβ1 tumors as compared to control tumors (Fig 2C). We further analyzed the in vivo migratory capacity using a xenograft model where tumor cells were injected in the yolk sac of zebrafish embryos and spreading throughout the embryo was quantified. Again, silencing β1 integrins led to an increased ability to migrate away from the primary tumor cell mass and travel to distant sites in the body (Fig 2D-F). When cells were injected into the developing blood system of these embryo's they rapidly became trapped in the vasculature and, in agreement with the migration patterns observed in 3D collagen matrices, wild type 4T1 cells showed cohesive outgrowth in those regions whereas 4T1shβ1 cells spread in a non-cohesive fashion (Fig S5).

We compared gene expression profiles in β1 knockdown and control 4T1 cells. Using a false discovery rate (FDR) <0.001 and 1.5-fold difference as a cut-of, 1230 differentially expressed genes were shared between both shβ1 lines (Table S1). In this set, Ingenuity Pathway Analysis predicted "*cellular movement*" as the process most significantly affected by β1 integrin silencing and also predicted *"cell-to-cell signaling"*. Both processes contained the *Cdh1* gene that was significantly downregulated in both shβ1 lines but not in the sh-control line, which was confirmed by qPCR (Fig 3A, S6). Indeed, silencing β1 integrins in 4T1 caused a

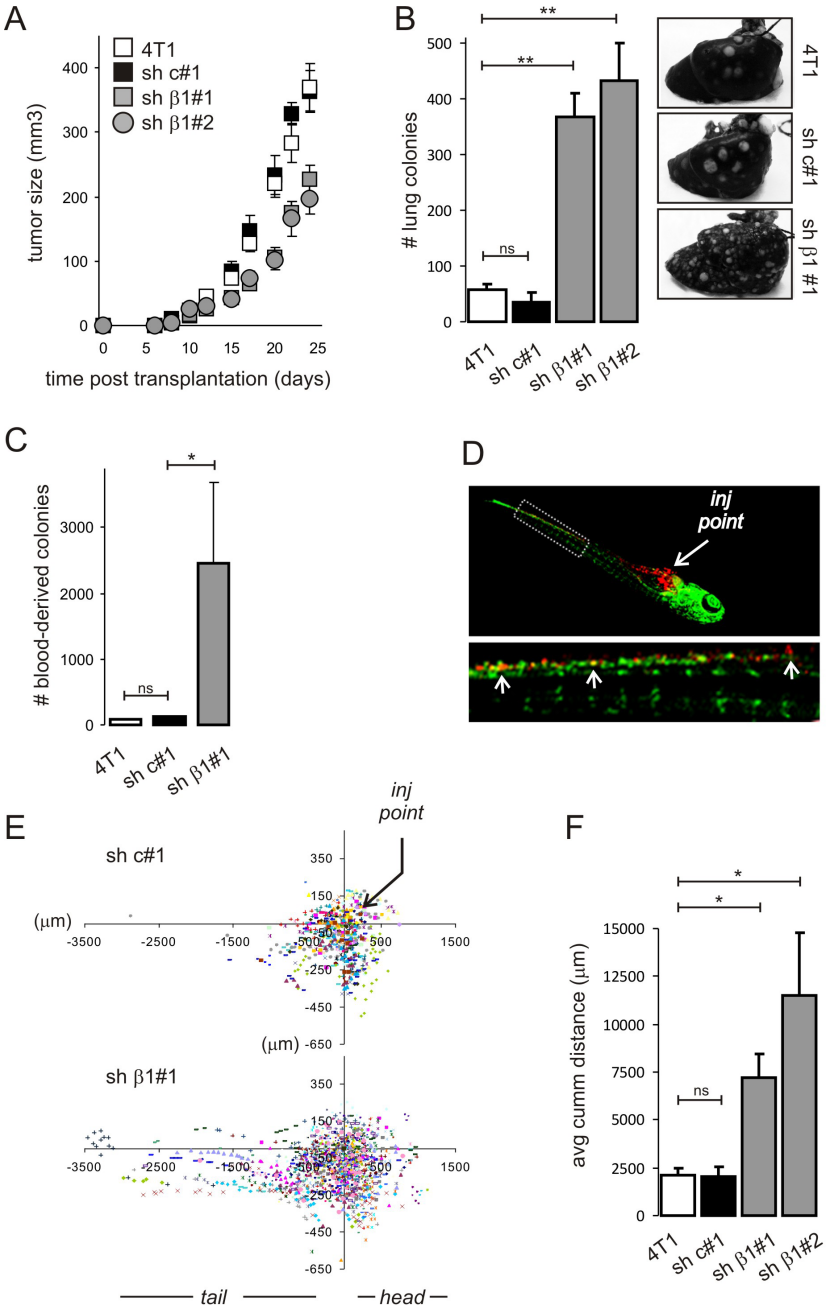

Figure 2. Integrin control of in vivo migration. A-C, primary tumor growth (**A**), spontaneous metastasis (**B**), and circulating tumor cells (**C**) following orthotopic transplantation of 4T1 cells expressing indicated shRNA constructs in mammary fat pad (>25 mice per condition from 3 independent experiments). **D**, CM-DiI labeled 4T1shβ1 cells injected in zebrafish yolk sac (top) and spread towards tail region 5 days post-injection (bottom). **E**, graphic representation of tumor cell spreading in ~40 zebrafish embryos per condition taken from 2 independent experiments (each pattern/color depicts tumor cells in a single embryo) in which labeled 4T1 cells expressing indicated shRNAs were injected in yolk sac. **F**, average of cumulative migration distances of tumor cells per embryo, calculated from E. *p<0.05; **p<0.001.

~75% reduction in E-cadherin surface expression and a similar trend was observed upon α2 silencing (Fig 3B). β1 silencing also diminished total E-cadherin protein levels and E-cadherin was reduced in primary tumors derived from 4T1shβ1 cells as compared to control tumors (Fig 3C,D). Notably, in agreement with their inability to switch to an individual cell migration mode (Fig 1A), silencing β1 integrins in MCF10a did not affect E-cadherin surface expression (Fig 3B).

There is evidence that integrin-mediated ECM adhesion can modulate cell-cell adhesion and both positive and negative regulation has been reported but crosstalk at the level of E-cadherin expression has not been demonstrated[9-11]. A very recent report showed that low levels of α2β1 integrin in human breast cancer are associated with poor survival[12]. This association was confirmed in the same "NKI 295" set but not in a larger, pooled breast cancer cohort from The Netherlands Cancer Institute, Amsterdam, NL[13] or in an independent breast cancer cohort from Erasmus University Rotterdam, NL[14] (not shown). Global expression of α2 or β1 was not associated with E-cadherin expression and associations detected in sub-groups were not corroborated in each cohort (not shown). This indicates that reduced expression of β1 integrins is not a general phenomenon in breast cancer, which may not be surprising given their dual role in growth and migration. Cell-ECM interactions can also be locally altered in tumors by changes in integrin activity or surface levels, or by altered proteolytic ECM degradation. Our findings indicate that such events can lead to transient E-cadherin downregulation in tumor cells already on the edge of EMT, allowing a subset of tumor cells to escape from the primary tumor mass and metastasize to distant organs.

We tested if the reduction in E-cadherin expression levels was critically involved in the pro-metastatic switch in cell migration strategy upon β1 integrin silencing. In support of this, ectopic expression of E-cadherin in 4T1shβ1 at similar surface levels as found in control 4T1 cells, restored cohesive invasion (Fig 3E, S7). It also blocked lung metastasis of 4T1shβ1 tumors (Fig 3F) whereas E-cadherin expression did not affect tumor growth, which was slow in β1 integrin-depleted tumors irrespective of the absence or presence of E-cadherin, further demonstrating that integrins control tumor growth and metastasis through separate pathways (Fig 3G).

Having established that the ability of β1 integrins to control E-cadherin levels can critically affect metastatic behavior, we investigated the mechanism by which β1 integrins control E-cadherin expression. Luciferase reporter assays showed that β1 integrin silencing led to a ~80% transcriptional downregulation of the *Cdh1* gene (Fig 4A). This prompted us to investigate regulation of a group of E-cadherin transcriptional repressors, including members of the Snail, bHLH, and ZFH families that are implicated in EMT[15]. Analysis of the micro-array data showed that of these repressors, only Zeb2 (also known as Sip1) was significantly and specifically upregulated in both 4T1shβ1 lines and qPCR confirmed the induction of Zeb2, but not Zeb1, upon β1 integrin silencing (Fig 4B, S6). ZEBs act as transcriptional repressors of miRNAs of the miR-200 family, which are expressed from two clusters on two distinct chromosomes. Vice versa, miR-200 family members post-transcriptionally repress Zeb1 and Zeb2 by targeting their 3' UTRs. This ZEB/miR-200 feedforward loop has been implicated in EMT, and alterations in the balance between ZEB and miR-200 may underlie progression of a number of different types of cancer, including breast carcinomas[16-18]. miRNA profiling indicated a strong downregulation of all five members of the miR-200 family in β1 integrin-

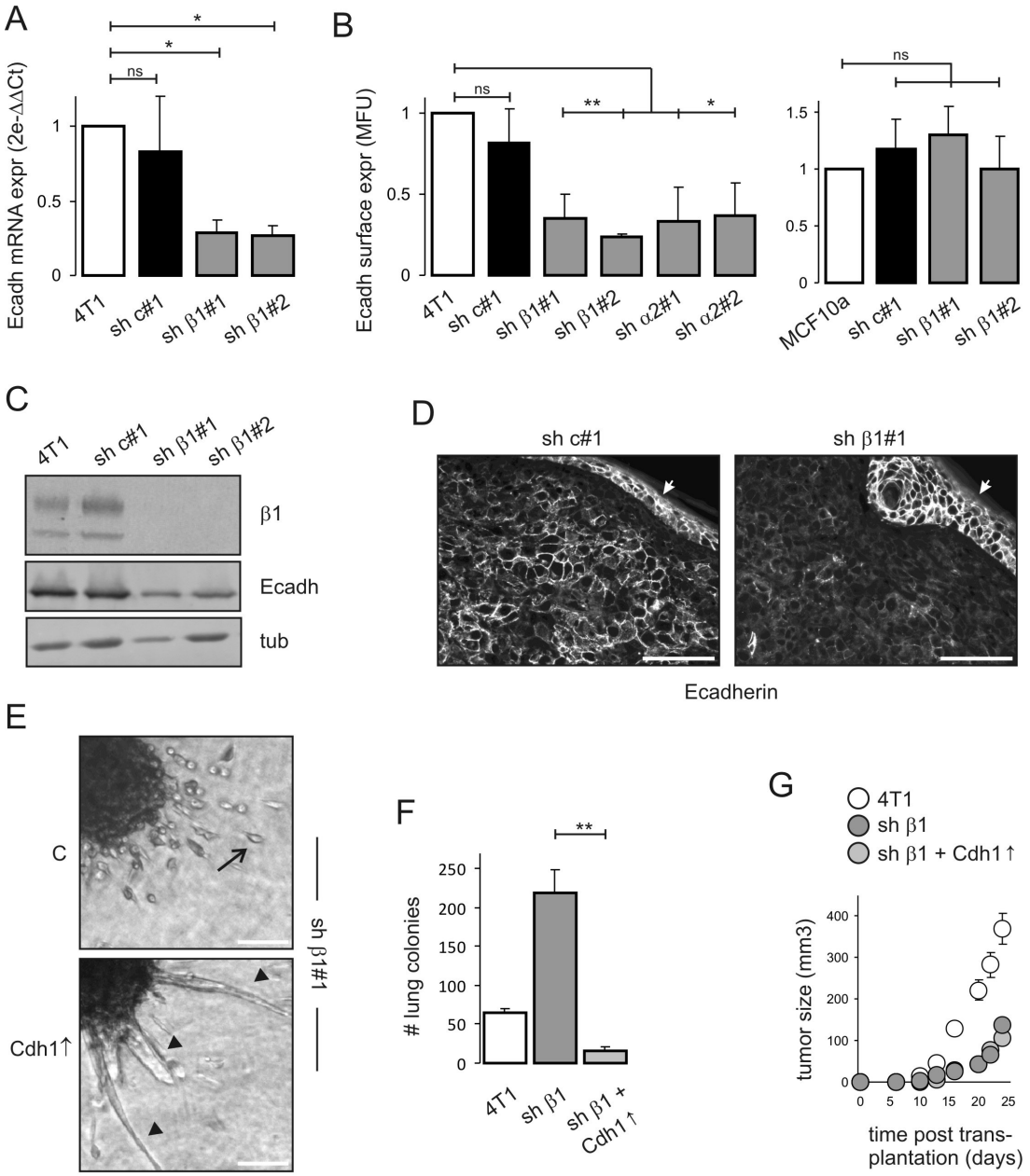

Figure 3. Suppression of E-cadherin is critical for integrin regulation of invasion and metastasis. A-C, E-cadherin mRNA (q-PCR) (**A**), surface expression (FACS) (**B**), and total protein (**C**) levels in 4T1 or MCF10a cells expressing indicated shRNAs. **D**, E-cadherin staining in 4T1 and 4T1shβ1 orthotopic breast tumors (arrow indicates skin, which serves as positive control in both). **E**, spheroids of 4T1shβ1 cells without (top) or with E-cadherin cDNA (bottom) 4 days post-injection in collagen gels (arrow indicates individual cell migration; arrow heads point to cohesive invasion strands). **F-G**, Lung metastasis (**F**) and primary tumor growth (**G**) of orthotopically transplanted 4T1 cells expressing indicated shRNAs and cDNAs (>20 mice per condition from 2 independent experiments). Scale bars, 50 μm. *p<0.05; **p<0.001.

depleted, but not control shRNA cells (Fig 4C).

We investigated the significance of miR-200 suppression for the observed β1 integrin-mediated control of the mode of invasion. Synthetic or lentiviral expression of any individual miR-200 family member restored cell-cell adhesion in 2D cultures of 4T1shβ1 cells and induced a reversal to cohesive 3D migration (Fig 4D, S8, S9, and data not shown). On the other hand, none of the miRNA hairpin inhibitors targeting miR-200 family members was able to interfere with cell-cell adhesion in wild type 4T1 cells (not shown). Together, these data point to overlapping functions of the miR-200 family members in this system and demonstrate that downregulation of all five members is required for the observed inhibition of cohesion upon depletion of β1 integrins. We noted that expression of each miR-200 expression construct was invariably lost within 5 days after GFP sorting, suggesting that the expression of mature miR-200 species caused a growth disadvantage (not shown). Finally, restored cell-cell adhesion upon expression of miR-200 family members in 4T1shβ1 cells was accompanied by a downregulation of Zeb2, concomitant with an upregulation of E-cadherin (Fig 4E), further pointing to a central role for Zeb2/miR-200 in β1 integrin-mediated control of E-cadherin based cohesion.

Altogether, our findings establish a novel connection between integrin-mediated cell-ECM interactions and E-cadherin-mediated adherens junctions. Interfering with β1 integrin-mediated ECM adhesion attenuates tumor growth but it can also disturb the ZEB/miR-200 balance leading to E-cadherin downregulation. This triggers a switch from cohesive to individual cell migration, which we find to act pro-metastatic in an orthotopic breast cancer model. The findings raise concerns with respect to the use of integrins as drug targets to sensitize tumors to radio- or chemotherapy. They also point to a new cross talk mechanism between cell-ECM and cell-cell adhesions that may regulate transient loss of cohesion in subpopulations of cancer cells within tumors where EMT has not been documented. While E-cadherin is almost invariably lost in invasive lobular breast carcinomas its expression is retained in many other types including the common ductal invasive carcinomas[19,20]. In those cases, a transient downregulation of E-cadherin through disturbed tumor cell-ECM interactions in a minor population of invading cells may drive metastasis while going unnoticed.

METHODS SUMMARY

4T1 mouse breast cancer cells and MCF10a human mammary epithelial cells were transduced using lentiviral shRNA or cDNA vectors and selected for integrin or E-cadherin surface expression by bulk FACS sorting. Synthetic miRNA Mimics and Hairpin Inhibitors were transfected at 50 nM, cells were replated next day, and used for FACS, qPCR, or collagen invasion 48 hours later. Cells expressing lentiviral miRNA shMIMICS were selected by bulk FACS sorting for GFP. E-cadherin firefly luciferase reporter[21] was transfected with CMV-renilla luciferase reporter and cells were analyzed using dual luciferase assay kit 3 days later. For 3D invasion, cell suspensions were microinjected into collagen gels followed by DIC imaging or immunostaining for F-actin or E-cadherin. Disintegrins and C-lectin type proteins, including Obustatin (α1β1), VLO4 (α5β1; αvβ3), VLO5 (α4β1; α9β1) and VP12 (α2β1), were used at 4.6 μM[5]. For orthotopic tumor growth, cells were injected into the fat pad of recipient mice

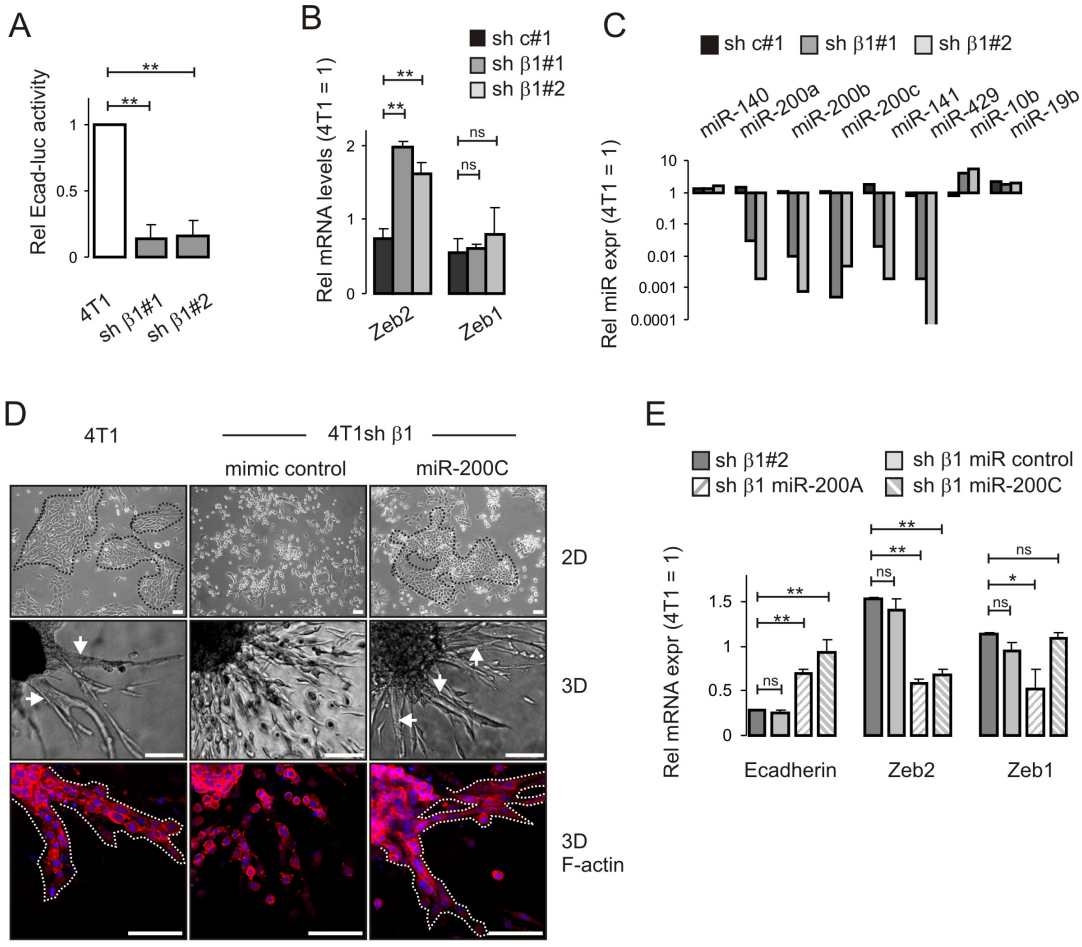

Figure 4. Integrin regulation of miR-200/ZEB balance controls E-cadherin expression and cohesive migration. A-B, E-cadherin promoter activity (luc assay) (**A**) and Zeb1 and Zeb2 mRNA levels (qPCR) (**B**) in 4T1 cells expressing indicated shRNAs. **C**, Relative levels of miRNAs in 4T1 expressing indicated shRNAs (qPCR array). **D**, 4T1 cohesion suppressed by shβ1 and restored by synthetic miR-200C Mimic in 2D culture (top) and in 3D collagen gels (middle and bottom) (arrows point to cohesive invasion strands). **E**, E-cadherin, Zeb2, and Zeb1 mRNA levels in 4T1shβ1 cells expressing indicated synthetic miRNA Mimics. Scale bars, 50 μm. *p<0.05; **p<0.001.

and tumor size was monitored. Affter 3-4 weeks, animals were anesthetized, primary tumor and lungs were excised for immunostaining and spontaneous metastasis counting, and in some cases blood was drawn for circulating tumor cells. For zebrafish xenotransplantation experiments, labeled tumor cells were injected into the yolk sac or developing vascular system of Fli-GFP transgenic zebrafish embryos. Embryos were maintained at 34°C for 6 days, and cumulative distance of spread tumor cells was calculated from confocal images. Real-time qPCR data were expressed using 2^(-ΔΔCt) method. Taqman microRNA qPCR assay kit was used for miRNA profiling. Affimetrix MG430 PM Array plates were used for micro-arrays. Differentially expressed genes were identified using random-variance t-test after quality

control and median normalization. Western blot and FACS were done as described[22]. Data are presented as mean ± SEM of at least 3 independent biological replicates unless otherwise stated. Student's t test (two-tailed) was used to compare groups.

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AUTHOR CONTRIBUTIONS

HT, VPSG, EN, and DvdH performed and analyzed in vitro experiments. HT, SELD, and RL performed and analyzed mouse experiments. VPSG and SH performed and analyzed zebrafish experiments. CM isolated disintegrins. EV and JHNM assisted in miRNA profiling and microarray data analysis. AA generated synthetic and lentiviral miRNA reagents. BvdW and BES were involved in discussions and writing of the manuscript. HT and EHJD planned experiments, discussed results, and wrote the manuscript. All authors critically read the manuscript. The authors declare no competing financial interests.

SUPPLEMENTARY TABLE, FIGURE, AND MOVIE LEGENDS

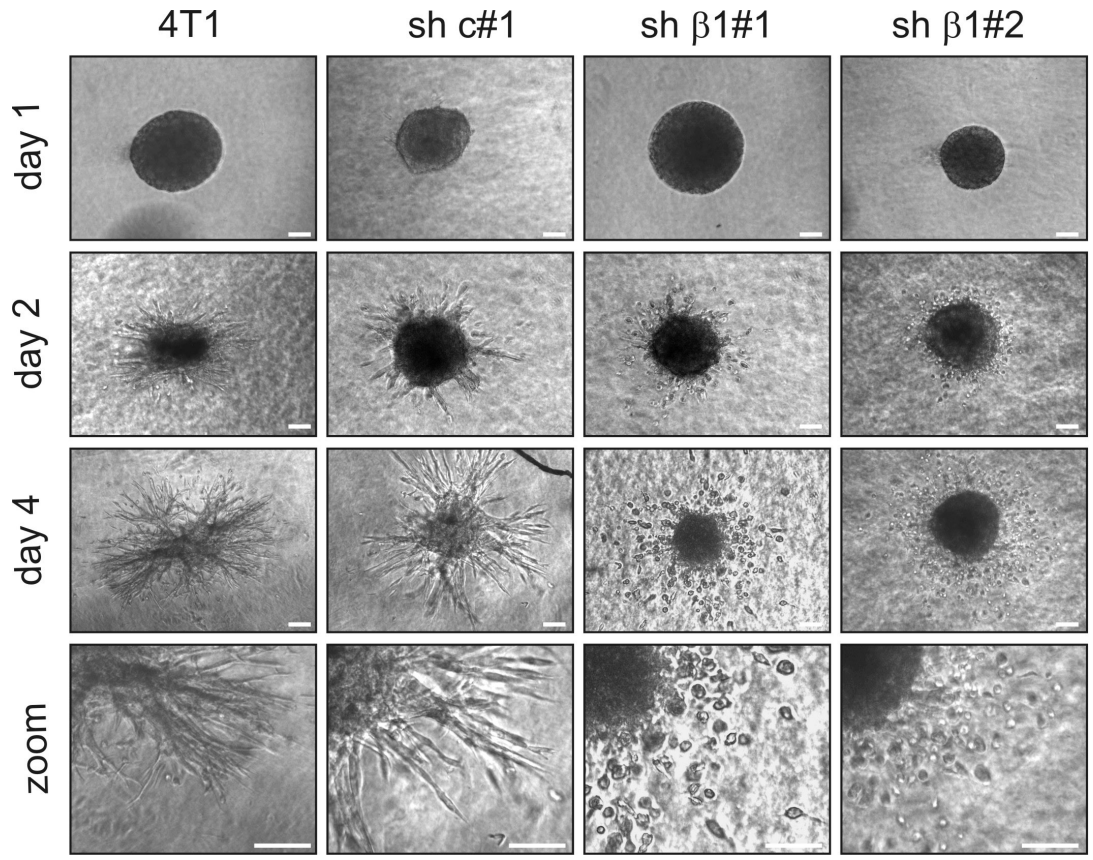

Figure S1. Spheroids of 4T1 cells expressing indicated shRNAs at indicated timepoints post-injection in collagen gels. Scale bars, 50 μm.

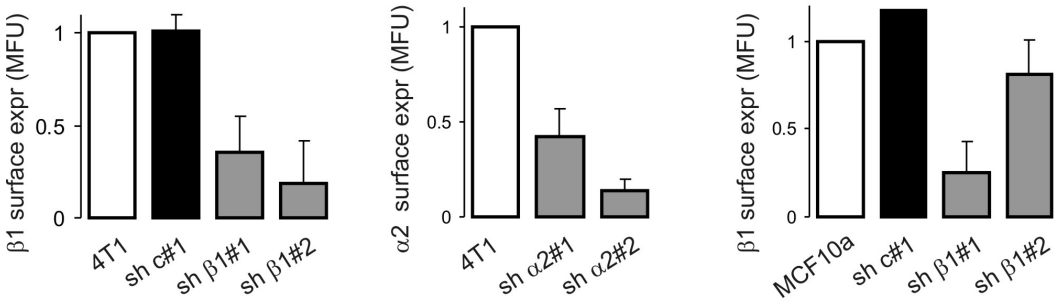

Figure S2. FACS analysis of β1 or α2 integrin surface expression in 4T1 or MCF10a cells expressing indicated shRNAs.

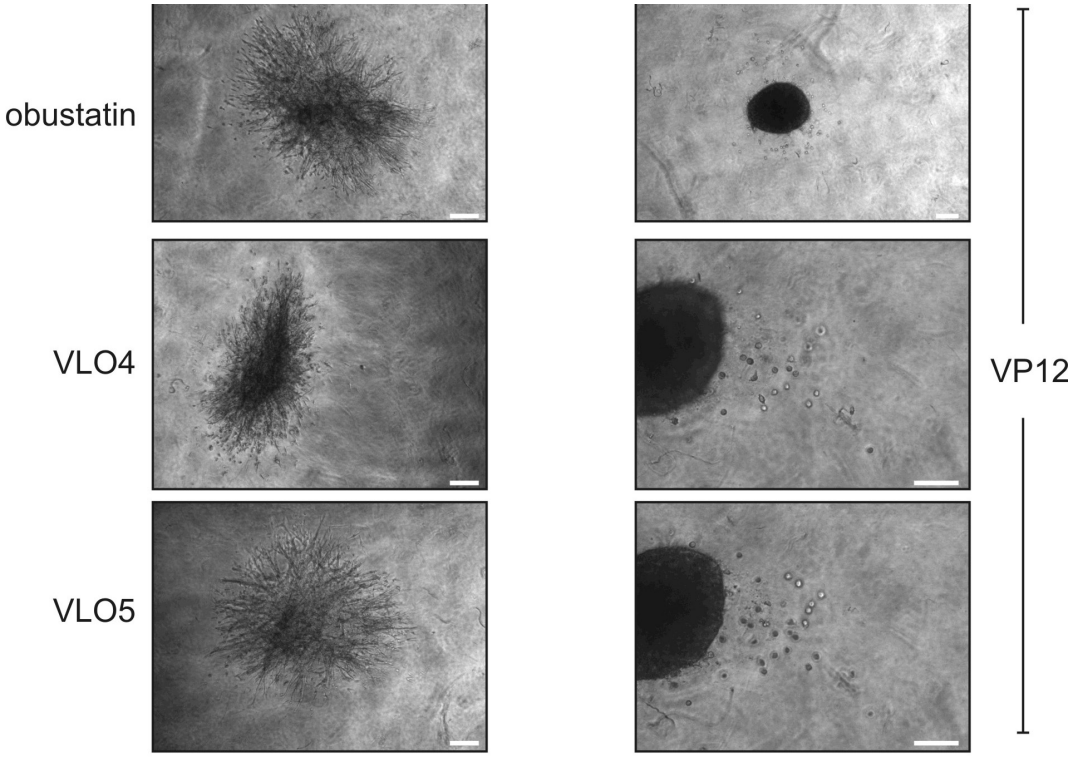

Figure S3. Spheroids of 4T1 cells in the presence of indicated peptides 4 days post-injection in collagen gels. Scale bars, 50 μm.

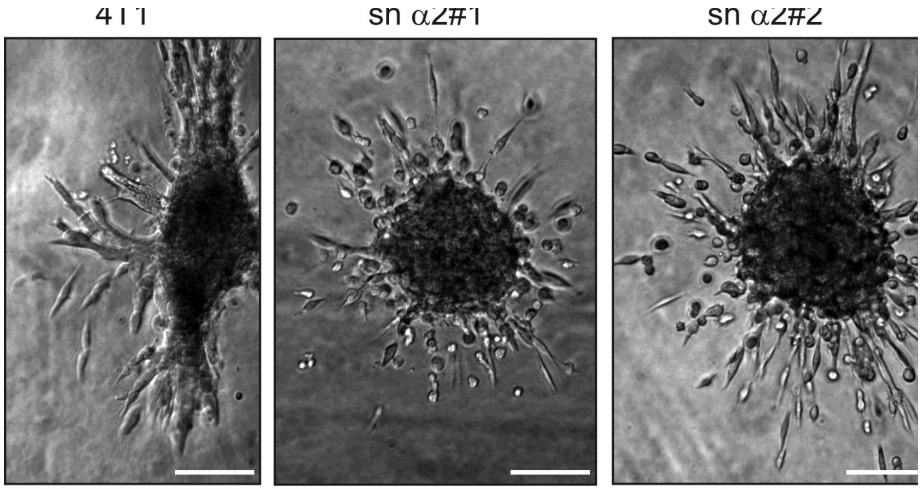

Figure S4. Spheroids of 4T1 cells expressing indicated shRNAs 4 days post-injection in collagen gels. Scale bars, 50 μm.

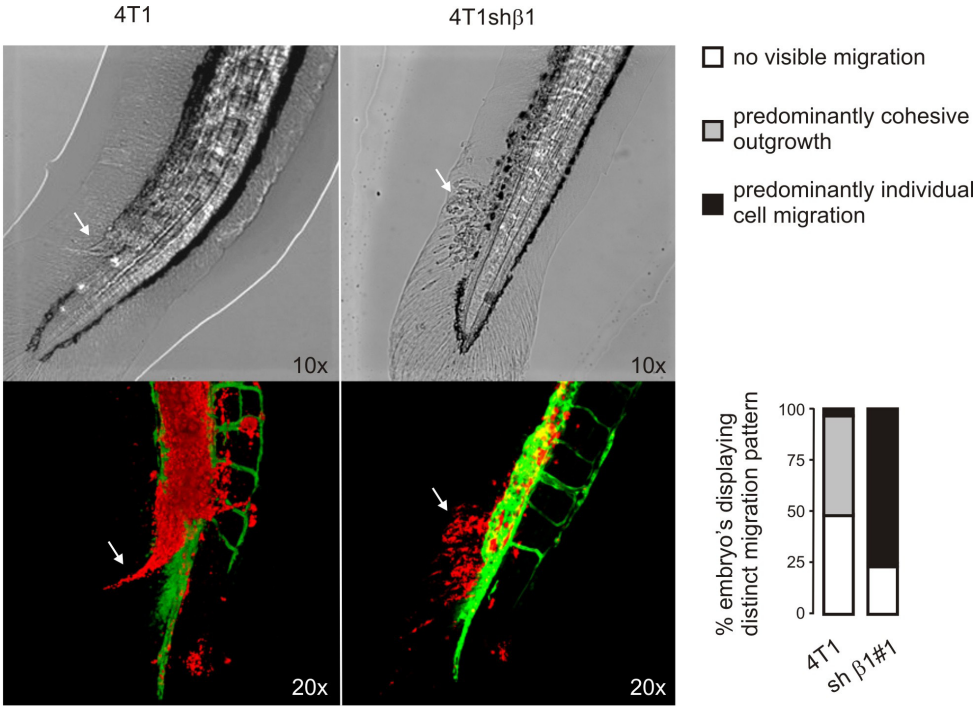

Figure S5. Cohesive outgrowth of wild type 4T1 cells (left image) versus non-cohesive spreading of 4T1shβ1 cells (right image) 3 days post-injection into the developing blood system of zebrafish embryos. DIC (top) and confocal fluorescent image stacks of bloodvessels (green) and labeled tumor cells (red) are shown. Graph shows % of injected embryos showing either of the two typical types of tumor cell migration patterns (15 embryos per condition analyzed).

	shβ1#1	shβ1#2
Cdh1	-4.1; 3.25e-10	-6.67; p=8.56e-11
Snail1	1.22; p=0.026	1.13; p=0.27
Snail2; Slug	1.10; p=0.35	1.02; p=0.73
Twist1	1.39; p=0.019	1.86; p=7.09e-8
Twist2	-1.13; p=0.022	-1.02; p=0.75
Zeb1	-1.03; p=0.57	1.21; p=0.0051
Zeb2	1.97; p=7.33e-5	2.45; p=2.56e-4
Tcf3; E2A; E12/E47	1.01; p=0.84	-1.05; p=0.55

Figure S6. Micro-array analysis of E-cadherin and indicated E-cadherin repressors in two independent 4T1shβ1 lines. Fold change compared to control group (4T1 wild type and 4T1 sh-control) and p-value is shown. Genes showing significant change (p<0.001) are indicated in blue.

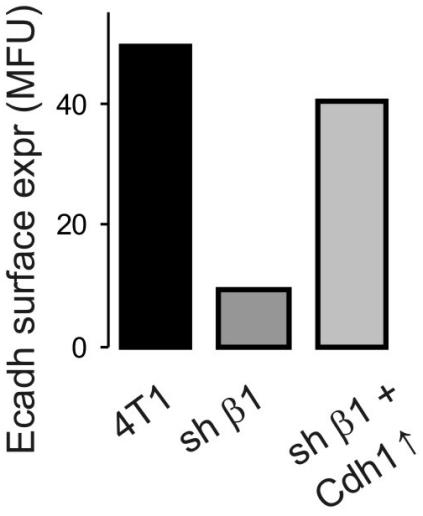

Figure S7. FACS analysis of E-cadherin surface expression in 4T1 cells expressing indicated shRNAs and cDNAs.

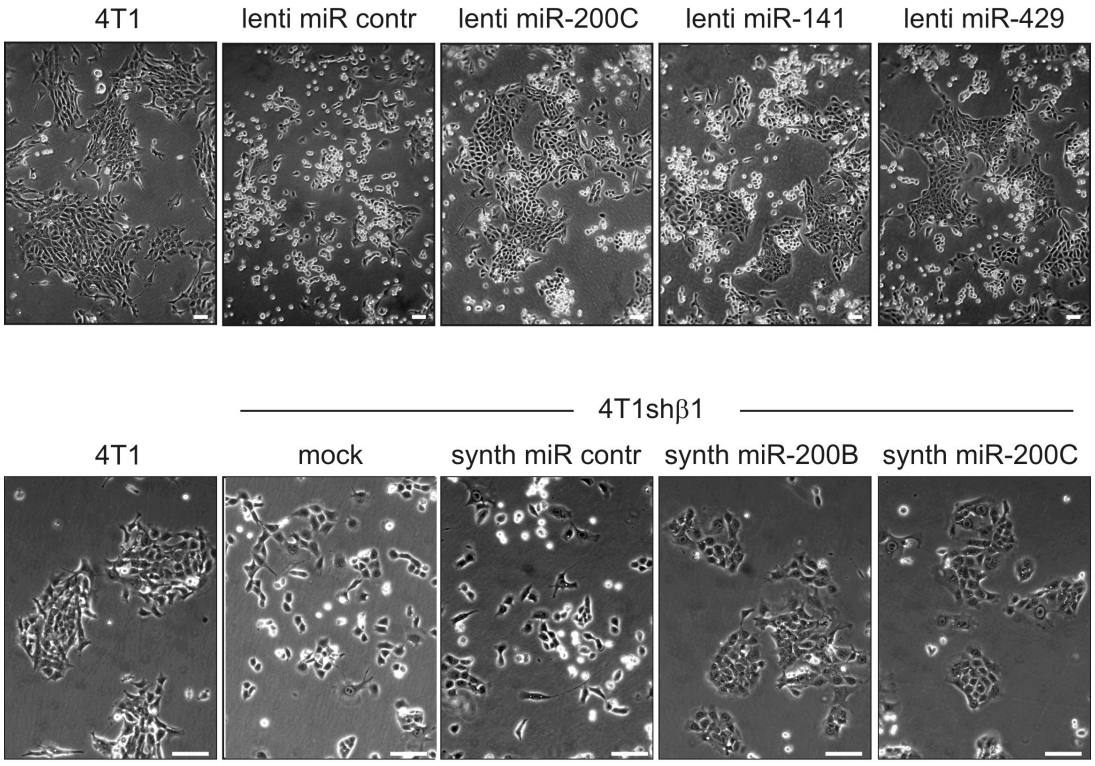

Figure S8. 4T1 cohesion in 2D culture suppressed by shβ1 and restored by lentiviral (top) or synthetic (bottom) expression of indicated miR-200 species. Scale bars, 50 μm.

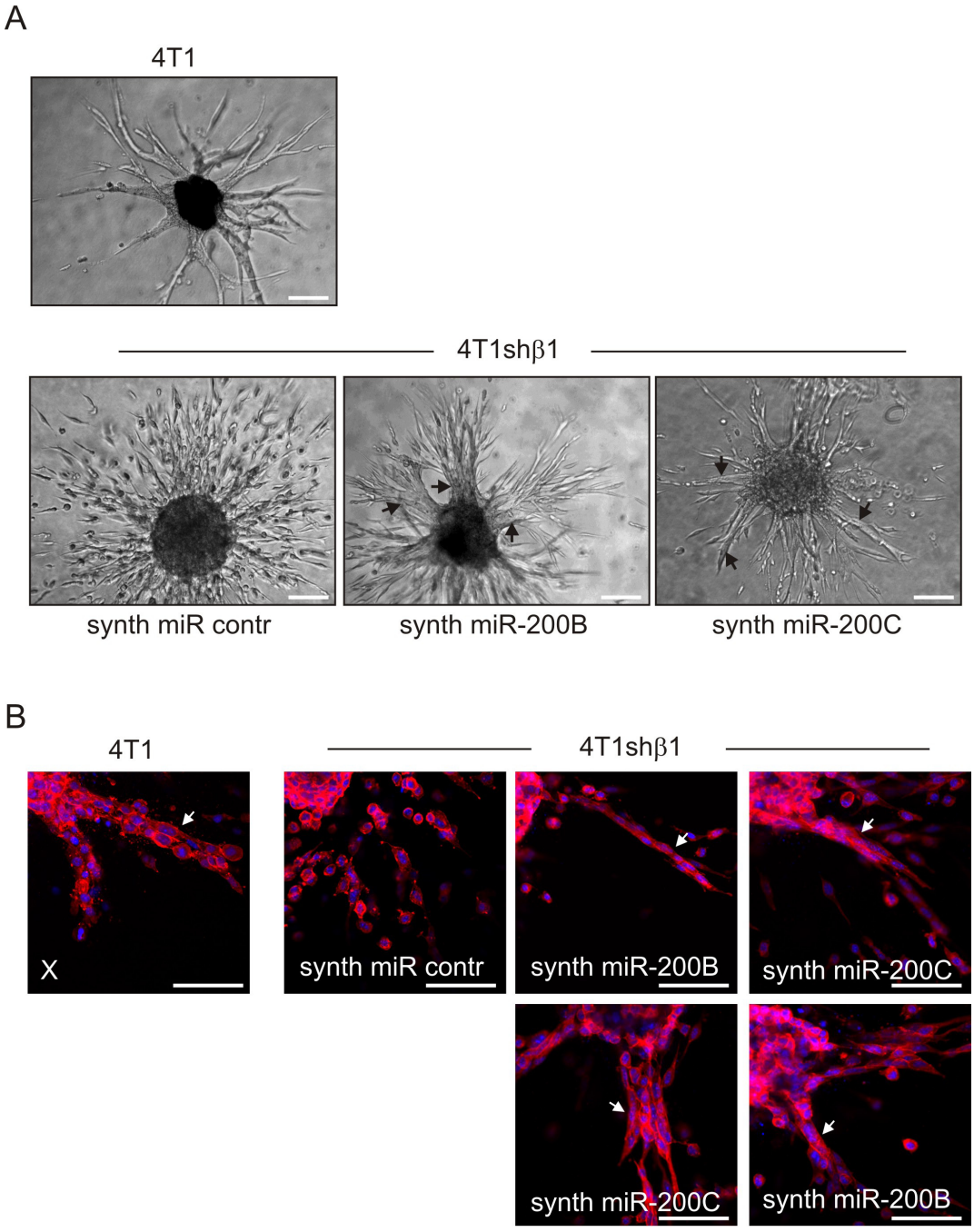

Figure S9. 4T1 cohesion in collagen gels suppressed by shβ1 and restored by synthetic expression of indicated miR-200 species. DIC (**A**) and actin & nuclear staining (Phalloidin & Hoechst; **B**) is shown. Arrows point to cohesive invasion strands. Scale bars, 50 μm.

Table S1: Differentially expressed genes shared between two independent 4T1shβ1 lines

Probe set	Gene symbol	fold-change shb1#1	fold-change shβ1#2
1428380_PM_at	0610007C21Rik	1.58	1.52
1429252_PM_at	0610010K14Rik	-1.81	-1.73
1447870_PM_x_at	1110002E22Rik	-2.06	-2.13
1435464_PM_at	1110003E01Rik	3.88	4.70
1442363_PM_at	1110012J17Rik	2.03	2.66
1434441_PM_at	1110018J18Rik	1.82	2.15
1431786_PM_s_at	1190003J15Rik	-2.64	-4.10
1429219_PM_at	1200009F10Rik	1.90	2.59
1428851_PM_at	1300014I06Rik	1.68	1.64
1442992_PM_at	130004C03	2.17	2.39
1417474_PM_at	1500035H01Rik	1.56	1.59
1439014_PM_at	1600021P15Rik	-1.51	-1.60
1429758_PM_at	1700017B05Rik	2.15	2.39
1423289_PM_a_at	1810029B16Rik	-1.52	-1.58
1454224_PM_at	2010300F17Rik	-3.26	-2.79
1424520_PM_at	2010305A19Rik	1.53	1.57
1453275_PM_at	2310002L13Rik	-16.31	-23.14
1428241_PM_at	2310035K24Rik	1.84	1.70
1429215_PM_at	2310058N22Rik	1.76	2.12
1428350_PM_at	2310061F22Rik	-2.14	-1.79
1428404_PM_at	2410025L10Rik	1.73	1.93
1434581_PM_at	2410066E13Rik	2.06	2.00
1436216_PM_s_at	2610204M08Rik	1.93	2.15
1426012_PM_a_at	2610301G19Rik	1.81	1.87
1429268_PM_at	2610318N02Rik	1.54	1.82
1424983_PM_a_at	2700078E11Rik	-1.73	-1.82
1418389_PM_at	2810453I06Rik	-1.98	-1.97
1432757_PM_at	2900011L18Rik	2.00	2.10
1444062_PM_at	2900056L01Rik	3.82	4.18
1436462_PM_at	3100002L24Rik	3.21	2.53
1452779_PM_at	3110006E14Rik	3.42	3.58
1458491_PM_at	4930422I07Rik	1.75	1.97
1441749_PM_at	4930447F24Rik	2.06	2.71
1435052_PM_at	4930455F23Rik	1.75	2.03
1429055_PM_at	4930506M07Rik	-1.72	-1.63
1454606_PM_at	4933426M11Rik	1.95	1.63
1432059_PM_x_at	5031425E22Rik	2.35	2.43
1430023_PM_at	5133400G04Rik	-1.54	-1.73
1435830_PM_a_at	5430435G22Rik	5.20	1.68
1454460_PM_at	5730433N10Rik	6.12	4.66
1441972_PM_at	6230424C14Rik	10.99	5.17
1439770_PM_at	6430598A04Rik	3.40	1.69
1454686_PM_at	6430706D22Rik	2.36	1.78
1433338_PM_at	6720460K10Rik	1.99	1.79
1429478_PM_at	6720463M24Rik	1.91	2.12
1428284_PM_at	8430427H17Rik	4.76	2.12
1430221_PM_at	9130008F23Rik	-3.27	-2.92
1415705_PM_at	9130011J15Rik	-1.61	-1.54
1436054_PM_at	9130227C08Rik	2.02	2.14
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1424829_PM_at	A830007P12Rik	2.41	2.80
1435169_PM_at	A930001N09Rik	1.89	1.72
1453062_PM_at	A930026I22Rik	1.76	2.00
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1456878_PM_at	AI646023	1.61	1.71
1455172_PM_at	AU020094	-2.04	-1.83
1452150_PM_at	AU040320	1.72	1.98
1455144_PM_s_at	AU040829	1.98	2.40
1458648_PM_at	AU042950	3.30	2.47
1423797_PM_at	Aacs	-1.72	-1.74
1451083_PM_s_at	Aars	-1.95	-1.72
1416402_PM_at	Abcb10	-1.66	-1.63
1428988_PM_at	Abcc3	4.06	3.46
1416014_PM_at	Abce1	-1.98	-1.66
1423570_PM_at	Abcg1	2.37	3.94
1439259_PM_x_at	Abhd4	1.73	1.52
1416946_PM_a_at	Acaa1a	2.44	2.93
1424184_PM_at	Acadvl	1.76	1.76
1439021_PM_at	Acap3	1.66	1.64
1424183_PM_at	Acat1	1.57	1.54
1425195_PM_a_at	Acat2	-1.58	-1.75
1429421_PM_at	Accs	1.66	1.63
1425326_PM_at	Acly	-2.55	-2.22
1436788_PM_at	Acp2	2.16	1.83
1456735_PM_x_at	Acpl2	2.83	3.12
1415873_PM_a_at	Actr1a	-1.57	-1.74
1419140_PM_at	Acvr2b	1.51	2.13
1417976_PM_at	Ada	-1.57	-1.90
1421172_PM_at	Adam12	-7.19	-11.30
1421858_PM_at	Adam17	1.56	1.70
1416871_PM_at	Adam8	-2.73	-2.23
1450716_PM_at	Adamts1	-9.42	-5.84
1437785_PM_at	Adamts9	6.21	7.75
1440668_PM_at	Adamtsl3	1.94	4.25
1423298_PM_at	Add3	-1.61	-1.85
1424393_PM_s_at	Adhfe1	2.77	1.70
1451992_PM_at	Adrbk1	-1.76	-1.79
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1416645_PM_a_at	Afp	3.54	3.63
1434287_PM_at	Agpat5	-1.76	-1.69
1426670_PM_at	Agrn	-1.58	-1.54
1422631_PM_at	Ahr	2.84	2.26
1422184_PM_a_at	Ak1	1.68	1.82
1457032_PM_at	Ak5	2.62	3.60
1434764_PM_at	Akap11	1.65	1.63
1419706_PM_a_at	Akap12	-2.79	-2.31
1455870_PM_at	Akap2	1.60	1.63
1433905_PM_at	Akap7	-1.87	-1.93
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1415776_PM_at	Aldh3a2	2.18	1.88	1418014_PM_a_at	B4galt1	1.62	1.64
1448104_PM_at	Aldh6a1	2.00	2.04	1425934_PM_a_at	B4galt4	-1.80	-1.53
1460167_PM_at	Aldh7a1	2.18	2.43	1433617_PM_s_at	B4galt5	1.95	2.10
1419115_PM_at	Alg14	-1.83	-1.56	1460329_PM_at	B4galt6	-11.53	-5.34
1455887_PM_at	Alg8	1.70	1.63	1438635_PM_x_at	B930041F14Rik	2.30	1.84
1434601_PM_at	Amigo2	13.54	10.99	1427947_PM_at	B9d2	2.20	1.98
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1434444_PM_s_at	Anapc1	1.79	1.55	1424360_PM_at	BC019943	-1.93	-1.70
1439066_PM_at	Angpt1	8.76	15.63	1455437_PM_at	BC033915	2.11	2.96
1448831_PM_at	Angpt2	1.99	1.54	1456929_PM_at	BC042782	2.42	4.11
1455090_PM_at	Angptl2	2.55	2.52	1435794_PM_at	BC050254	1.66	1.70
1453287_PM_at	Ankrd33b	2.76	5.11	1424117_PM_at	BC056474	-1.56	-1.53
1451182_PM_s_at	Ankrd54	1.68	1.60	1435209_PM_at	BC057079	-1.77	-1.53
1451446_PM_at	Antxr1	4.84	4.02	1424951_PM_at	Baiap2l1	-1.75	-1.79
1418468_PM_at	Anxa11	1.93	2.16	1453076_PM_at	Batf3	1.64	1.79
1460330_PM_at	Anxa3	-1.59	-1.50	1450622_PM_at	Bcar1	-1.89	-2.16
1416137_PM_at	Anxa7	1.52	1.74	1416647_PM_at	Bckdha	2.22	2.27
1460190_PM_at	Ap1m2	-3.42	-3.22	1419406_PM_a_at	Bcl11a	-2.40	-2.77
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1422593_PM_at	Ap3s1	2.04	1.80	1452614_PM_at	Bcl2l15	-1.95	-2.60
1449070_PM_x_at	Apcdd1	7.71	13.26	1442187_PM_at	Bdkrb2	-2.83	-2.74
1456500_PM_at	Aph1b	1.78	2.27	1422169_PM_a_at	Bdnf	2.70	4.76
1416203_PM_at	Aqp1	23.48	9.06	1426489_PM_s_at	Bfar	1.55	1.63
1418818_PM_at	Aqp5	7.03	2.53	1418025_PM_at	Bhlhe40	-4.86	-7.41
1452291_PM_at	Arap2	-2.92	-8.37	1425532_PM_a_at	Bin1	1.82	1.82
1421134_PM_at	Areg	2.45	2.39	1417691_PM_at	Bin3	2.22	1.99
1418847_PM_at	Arg2	2.58	3.03	1426238_PM_at	Bmp1	1.58	1.52
1426952_PM_at	Arhgap18	6.87	5.56	1418910_PM_at	Bmp7	-3.06	-2.58
1435108_PM_at	Arhgap22	2.10	2.17	1416923_PM_a_at	Bnip3l	1.57	1.73
1435694_PM_at	Arhgap26	1.87	2.05	1435480_PM_at	Braf	-2.09	-2.06
1434809_PM_at	Arhgap28	3.07	3.10	1448521_PM_at	Brd7	-1.67	-1.51
1454745_PM_at	Arhgap29	-2.49	-1.86	1427270_PM_a_at	Bsdc1	1.73	1.86
1451867_PM_x_at	Arhgap6	5.99	4.61	1435249_PM_at	Btaf1	-2.03	-1.70
1448660_PM_at	Arhgdig	-2.25	-1.58	1424054_PM_at	Btbd2	2.05	1.63
1421164_PM_a_at	Arhgef1	-1.51	-1.83	1417987_PM_at	Btd	1.83	1.96
1424250_PM_a_at	Arhgef3	-1.95	-3.81	1424074_PM_at	Btf3l4	1.67	2.07
1431429_PM_a_at	Arl4a	1.86	1.75	1426268_PM_at	C130090K23Rik	-2.05	-2.64
1427167_PM_at	Armcx4	-2.59	-3.90	1422772_PM_at	C1galt1	-1.93	-1.86
1454617_PM_at	Arrdc3	7.73	7.49	1435580_PM_at	C230081A13Rik	2.74	3.75
1434961_PM_at	Asb1	2.34	1.82	1436709_PM_at	C230096C10Rik	1.79	1.83
1423422_PM_at	Asb4	-3.38	-3.65	1457046_PM_s_at	C77370	-1.89	-1.94
1451095_PM_at	Asns	-2.35	-1.87	1446288_PM_at	C78692	2.15	2.13
1448763_PM_at	Atad1	-1.78	-1.99	1441673_PM_at	C80120	1.75	2.20
1449363_PM_at	Atf3	-3.57	-3.31	1440513_PM_at	C80258	1.91	2.51
1451747_PM_a_at	Atg12	2.00	1.73	1453232_PM_at	Calr3	1.71	1.98
1434092_PM_at	Atg9b	-2.76	-3.27	1452050_PM_at	Camk1d	3.29	6.73
1456388_PM_at	Atp11a	-1.61	-1.59	1439168_PM_at	Camk2d	1.82	1.55
1452746_PM_at	Atp13a2	1.53	1.62	1423941_PM_at	Camk2g	2.39	1.92
1436921_PM_at	Atp7a	2.15	2.12	1439843_PM_at	Camk4	2.00	2.73
1418774_PM_a_at	Atp7a	2.15	2.00	1426901_PM_s_at	Camta2	1.92	2.03
1434026_PM_at	Atp8b2	2.17	1.98	1437537_PM_at	Casp9	1.75	1.86
1415932_PM_x_at	Atp9a	1.73	2.00	1449145_PM_a_at	Cav1	-2.48	-2.66
1453681_PM_at	Atpif1	1.69	1.94	1422666_PM_at	Cblc	-1.73	-2.16

Movie S1. 4T1 cell migration in collagen gel (start 48h post injection; duration ~3h).

Movie S2. 4T1shβ1 cell migration in collagen gel (start 48h post injection; duration ~3h).

Movie S3. 4T1 cell migration in collagen gel incubated with VP12 (start 48h post injection; duration ~3h).

FULL METHODS

Cell lines and animals. 4T1 mouse breast cancer cells and MCF10a human mammary epithelial cells were obtained from ATCC and cultured according to the provided protocol. Rag2−/−;γc−/− mice were housed in individually ventilated cages under sterile conditions. Housing and experiments were performed according to the Dutch guidelines for the care and use of laboratory animals. Sterilized food and water were provided ad libitum. Zebrafish were maintained according to standard protocols (http://ZFIN.org). Embryos were grown at 28.5-30˚C in egg water (60 μg/ml Instant Ocean Salts). During injection with tumor cells, embryos were kept under anesthesia in 0.02% buffered 3-aminobenzoic acid ethyl ester (Tricaine, Sigma).

Antibodies and peptides. For FACS, primary antibodies included HMβ1 anti-mouse β1 (BD Pharmingen), AIIB2 anti-human β1, Ha1/29 anti-mouse α2 (BD Pharmingen), or DECMA anti-mouse/human E-cadherin (Sigma-Aldrich). For Western blot, primary antibodies included HMβ1 anti-mouse β1, 36/E-cadh anti-mouse/human E-cadherin (BD Transduction Laboratories), and B-5-1-2 anti-α-tubulin (Sigma). For immunohistochemistry on frozen tumor sections and in fixed collagen gels, 36/E-cadh anti-mouse/human E-cadherin antibody (BD Transduction Laboratories) was used. For tumor cell migration interference studies, snake venom-derived disintegrins and C-lectin type proteins, including Obustatin (α1β1), VLO4 (α5β1; αvβ3), VLO5 (α4β1; α9β1) and VP12 (α2β1), were used at a concentration of 4.6 μM[5].

Stable cDNA and shRNA expression. 4T1 and MCF10a cells were transduced using lentiviral shRNA vectors (LentiExpress™; Sigma-Aldrich) according to the manufacturers' procedures and selected in medium containing 2 μg/ml puromycin. Control vectors included shRNA targeting TurboGFP (shc#1) and shRNA targeting eGFP (shc#2). shRNAs silencing mouse β1 integrin included those targeting *gcacgatgtgatgatttagaa* (shβ1#1; nucleotides 360-383 in the mouse *Itgb1* coding sequence) and *gccattactatgattatcctt* (shβ1#2; nucleotides 1111-1131 in the mouse *Itgb1* coding sequence). shRNAs silencing mouse α2 integrin included those targeting *gcgttaattcaatatgccaat* (shα2#1; nucleotides 733-753 in the mouse *Itga2* coding sequence) and *gcagaagaatatggtggtaaa* (shα2#2; nucleotides 2274-2294 in the mouse *Itga2* coding sequence). shRNAs silencing human β1 integrin included those targeting *gccctccagatgacatagaaa* (shβ1#1; nucleotides 360-380 in the human *ITGB1* coding sequence) and *gccttgcattactgctgatat* (shβ1#2; nucleotides 2367-2387 in the human *ITGB1* coding sequence). 4T1shβ1 cells were transduced with pCSCG/mECAD lentiviral cDNA expression vector for mouse E-cadherin (provided by Dr. Patrick Derksen, University Medical Center, Utrecht NL). Cells transduced with integrin shRNAs or E-cadherin cDNA were selected for

stable knockdown or stable expression phenotypes, respectively by two rounds of bulk FACS sorting (see below for technical details).

Lentiviral expression of miRNA shMimics.
4T1shβ1 cells were transduced using miRIDIAN shMIMIC Lentiviral miRNAs (non-targeting control, miR-200a, miR-200b, miR-200c, miR-141, and miR-205; ThermoFisher Scientific) according to the manufacturers' procedures followed by two rounds of bulk sorting for GFP expression. Subsequently, cells were used for E-cadherin FACS, qPCR analysis, or collagen invasion studies.

Transfection of synthetic miRNA Mimics, and miRNA Hairpin Inhibitors.
Cells were seeded at 5x105 cells per well in 12 wells plates and transfected at a final concentration of 50 nM of miRIDIAN miRNA Mimics (ThermoFisher Scientific; control non-targeting, miR-200a, miR-200b, miR-200c, miR-141, and miR-205), or miRIDIAN miRNA Hairpin Inhibitors (ThermoFisher Scientific; control non-targeting, miR-200a, miR-200b, miR-200c, miR-141, and miR-205) using DharmaFECT2 (ThermoFisher Scientific). Cells were replated 24 hours post transfection and used for E-cadherin FACS, qPCR analysis, or collagen invasion 48 hours later.

Luciferase reporter assay. 4T1 wild type and 4T1shβ1 cells were transiently transfected with 10 ng of an E-cadherin firefly luciferase reporter plasmid[21] (provided by Dr. Geert Berx, VIB, Gent BE) and 2 ng of a CMV-renilla luciferase reporter using lipofectamine plus (Invitrogen) and analyzed using a dual luciferase kit (Promega) 3 days later, according to the manufacturers' procedure.

3D invasion assays. Cell suspensions in PBS containing 2% polyvinylpyrrolidone (PVP; Sigma-Aldrich) were microinjected (~$1x10^4$ cells/droplet) using an air driven microinjector (20 psi, PV820 Pneumatic PicoPump; World precision Inc) into collagen gels prepared from 2.5 mg/ml acid-extracted rat tail collagen type 1. Tumor cell spheroids were monitored for 4 days. For immunostaining at 4 days post-injection, gels were incubated for 30 min with 5 µg/ml collagenase (from Clostridium histolyticum, Boehringer Mannheim) at room temperature, fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 1% BSA. Gels were incubated with Rhodamin-conjugated Phalloidin or with E-cadherin antibody followed by Alexa 488-conjugated secondary antibody and Hoechst nuclear staining. Preparations were mounted in Aqua-Poly/Mount solution (Polysciences, Inc) and analyzed using a Nikon TE2000 confocal microscope. Z-stacks (50 x 1 µm) were obtained using a 20x dry objective and converted into a single Z projection using the "extended depth of field" plugin from ImageJ software.

For real time imaging, ~3 hours time-lapse movies of spheroids were obtained starting at 48 hours post-injection. Image acquisition was performed using a Nikon TE2000 confocal microscope with a temperature and CO_2 controlled incubator. Differential interference contrast (DIC) time-lapse videos were recorded using a charged coupled device (CCD) camera controlled by NIS Element Software. Images were converted into a single avi file in Image-Pro Plus (Version5.1; Media Cybernetics).

Mouse orthotopic transplantation experiments. $1×10^5$ tumor cells in 0.1 mL PBS were injected

into the fat pad of 8-12-week old female Rag2$^{-/-}$;γc$^{-/-}$ mice. Size of the primary tumors was measured using calipers. Horizontal (*h*) and vertical (*v*) diameters were determined and tumor volume (*V*) was calculated: $V = 4/3\pi\{1/2[\sqrt{}(h \times v)]^3\}$. After 3-4 weeks, animals were anesthetized with pentobarbital and primary tumor and lungs were excised. Primary tumor and left lung were divided into two pieces that were snap frozen in liquid nitrogen for E-cadherin immunostaining or fixed in 4% paraformaldehyde for H&E staining. For counting of lung metastases, right lungs were injected with ink solution, destained in water, and fixed in Feketes [4.3% (vol/vol) acetic acid, 0.35% (vol/vol) formaldehyde in 70% ethanol]. To analyze circulating tumor cells in some mice blood was drawn from the right atrium via heart puncture after anesthetizing but before excision of primary tumor and lungs. 0.2 ml of blood was plated into 60-mm tissue culture dishes filled with growth medium. After 5 days, tumor cell clones were stained using MTT (Sigma) and counted using ImageJ.

Zebrafish xenotransplantation experiments. For quantification of tumor cell spreading, tumor cells were labeled with CM-DiI (Invitrogen), mixed with 2% PVP, and injected into the yolk sac of enzymatically dechorionated, two-day old Fli-GFP transgenic zebrafish embryos using an air driven microinjector (20 psi, PV820 Pneumatic PicoPump; World precision Inc). Embryos were maintained in egg water at 34°C for 6 days and subsequently fixed with 4% paraformaldehyde. Imaging was done in 96 well plates containing a single embryo per well using a Nikon Eclipse Ti confocal laser-scanning microscope. Z stacks (15 x 30 μm) were obtained using a Plan Apo 4X Nikon dry objective with 0.2 NA and 20 WD. Images were converted into a single Z projection in Image-Pro Plus (Version 6.2; Media Cybernetics). Automated quantification of cumulative tumor cell spreading per embryo was carried out using an in-house built Image-Pro Plus plugin.
For analysis of tumor cell migration strategies, labeled tumor cells were injected in the developing blood system and clusters of cells trapped in the vasculature were imaged 3 days post injection. Z stacks (75 x 1 μm) were obtained using a Plan Apo 20X Nikon dry objective with 0.2 NA and 20 WD.

mRNA and miRNA analysis. Total RNA for qPCR and miRNA profiling was extracted using Trizol (Invitrogen). cDNA was randomly primed from 50 ng total RNA using iScript cDNA synthesis kit (BioRad) and real-time qPCR was subsequently performed in triplicate using SYBR green PCR (Applied Biosystems) on a 7900HT fast real-time PCR system (Applied Biosystems). The following qPCR primer sets were used: β-actin, forward *aacctggaaaagatgacccagat* reverse *cacagcctggatggctacgta*; E-cadherin, forward *atcctcgccctgctgatt* reverse *accaccgttctcctccgta*; Zeb1, forward *ccttcaagaaccgctttctgtaaa* reverse *cataatccacaggttcagttttgatt*; Zeb2, forward *cagcagcaagaaatgtattggtttaa*, reverse *tgtttctcattcggccatttact*. Data were collected and analysed using SDS2.3 software (Applied Biosystems). Relative mRNA levels after correction for β-actin control mRNA, were expressed using 2^(-ΔΔCt) method.

Detection of mature miRNAs was performed using Taqman microRNA qPCR assay kit according to the manufacturer's instructions (Applied Biosystems). The U6 small nuclear RNA was used as internal control.

For micro-arrays, total RNA was extracted using mirVana RNA isolation kit (Ambion Inc). RNA quality and integrity was assessed with Agilent 2100 Bioanalyzer system (Agilent technologies). The Affymetrix 3' IVT-Express Labeling Kit was used to synthesize Biotin-

labeled cRNA and this was hybridized to an Affimetrix MG430 PM Array plate. Data quality control was performed with Affymetrix Expression Console v1.1 and all raw data passed the affimetrix quality criteria. Median normalization of raw expression data and identification of differentially expressed genes using a random-variance t-test was performed using BRB ArrayTools[23] version 4.1.0 Beta 2 release (developed by Dr. Richard Simon and BRB-ArrayTools Development Team members; http://linus.nci.nih.gov/BRB-ArrayTools.html). Annotation was done according to the NetAffx annotation date release 2009-11-23. Corrections for multiple testing were performed as described by calculating the FDRs[24].

Western blot and flow cytometry. For Western blot, cells were lysed with modified RIPA buffer (150 mM NaCl, 1.0% triton-X 100, 0.5% Na deoxycholate, 0.1% 50mM Tris pH 8, and protease cocktail inhibitor (Sigma-Aldrich)). Samples were separated on SDS PAGE gels and transferred to PVDF membranes (Millipore), incubated with primary antibodies followed by horseradish peroxidase-labeled secondary antibodies (Jackson ImmunoResearch Laboratories inc), and developed with enhanced chemiluminescence substrate mixture (ECL plus, Amersham, GE Healthcare). Blots were scanned on a Typhoon 9400 (GE Healthcare).

For flowcytometry, cells were detached either using trypsin/EDTA (in the case of GFP or integrin surface expression) or by 0.02% EDTA only (in the case of E-cadherin surface expression). Surface expression levels were determined using primary antibodies, followed by fluorescence-conjugated secondary antibodies, and analysis on a FACSCanto or sorting on a FACSCalibur (Becton Dickinson).

Statistical analysis. Data are presented as mean ± SEM of at least 3 independent biological replicates unless otherwise stated. Student's t test (two-tailed) was used to compare groups.

References to full methods.

23. Wright G.W. and Simon R. (2003) A random variance model for detection of differential gene expression in small microarray experiments. *Bioinformatics* 19:2448-2455.
24. Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Royal Stat Soc*, Series B (Methodological) 57:289–300.

Summary and discussion

Summary and discussion

Embryo development, wound healing, angiogenesis, and cancer metastasis rely on cell motility and adhesion dynamics. This involves shifting integrin expression profiles, which may reflect the changing environment that the cells encounter and adapt to. For example, malignant cells migrating from primary to secondary sites will come across extracellular matrix (ECM) compositions that are different from their site of origin. In ***chapters 2 and 3*** of this thesis, the roles of $\alpha5\beta1$ and $\alpha v\beta3$ fibronectin (FN)-binding integrins in cell motility and adhesion dynamics are discussed. We find that when these integrins are ectopically expressed in the same cellular background (GE11), striking differences in cellular functions (cell morphology, cell-matrix adhesion dynamics and localization) are observed. Cells expressing $\alpha5\beta1$ integrin exhibit a contractile fibroblastic morphology with highly dynamic centripetally orientated cell-matrix adhesions and migrate in random fashion. In contrast, $\alpha v\beta3$ expressing cells, whose cell-matrix adhesions are more static and distributed across the basal surface, migrate in a highly persistent fashion.

What is the reason for these differences when both integrins interact with FN? One possible explanation could be the interaction of signaling and/or adaptor proteins with specific residues within the integrin cytoplasmic tail. However, α- and β- tail swapping experiments revealed that this is probably not the case. Rather, our evidently suggests that it is how the integrins interact with the FN that affects cell signaling. We demonstrate that both $\alpha5\beta1$ and $\alpha v\beta3$ adhere to immobilized (stretched) FN, but only $\alpha5\beta1$ binds to soluble folded (inactive) FN. RGD in immobilized (stretched) FN is reoriented in such a way that it is accessible to both integrins (Altroff et al., 2004). The ability of $\alpha5\beta1$ to access the RGD site in soluble FN comes from the fact that this integrin has an additional FN binding region (Aota, Nomizu, and Yamada, 1994; Bowditch et al., 1994; Danen et al., 1995) and we show that placing the CTSEQNC hypervariable sequence in the I-like domain of $\beta1$ in the context of $\beta3$ allows $\alpha v\beta3$ to also bind soluble FN. It has been suggested that the $\alpha5\beta1$ hypervariable sequence binds to the PHSRN “synergy” region in IIIFN9 to stabilize interaction with the RGD region in IIIFN10 by changing the tilt angle between IIIFn10 and IIIFn9, subsequently exposing RGD loops. Our findings demonstrate that integrin $\alpha v\beta3$, even if locked in a high affinity state by different mutations, binds poorly to soluble FN because it lacks

this functionality (Danen et al., 1995; Sechler, Corbett, and schwarzbauer, 1997).

The association with the synergy region may explain the apparently specific ability of $\alpha5\beta1$ to form a "catch-bond" with FN and mediate adhesion strengthening (Friedland, Lee, and Boettiger, 2009; Roca-Cusachs et al., 2009). Importantly, our findings indicate that switching between these two integrins with such distinct ligand-interaction modes strongly affects intracellular cellular with effects on RhoA activity, cytoskeletal contractility, and ECM assembly.

In ***chapter 4***, a novel $\alpha v\beta3$ binding partner is described that could also contribute to the specific $\alpha v\beta3$-mediated effects on cell morphology. MacMarcks (MRP) had been implicated in the activation of integrins and cell spreading by regulating the cortical actin network (Jin and Li, 2002). Interestingly, expression of $\beta3$ integrin transcriptionally down-regulates MRP. We demonstrate that the region in the vicinity of NITY domain of the $\beta3$ tail down-regulates MRP if associated with the αv-subunit. Nonetheless, silencing MRP did not promote cell spreading in the parental line, and overexpression did not hamper cell spreading in cells using $\alpha v\beta3$ for adhesion. This suggests that $\beta3$ is required for MRP localization and expression but MRP is not essential for $\alpha v\beta3$-mediated cell spreading (in contrast to $\beta2$ integrins, which have been claimed to depend on MRP for spreading (Li et al., 1996).

As described in ***chapter 5***, certain diseases display altered expression or functionality of integrins. For instance, high expression levels of various types of integrins have been correlated with tumor progression in a numbers of cancers (Mizejewski, 1999). For that reason, antagonists such as peptidomimetics and monoclonal antibodies have been developed targeting either $\alpha5\beta1$ and $\alpha v\beta3$ integrins. Disintegrins are RGD-containing cysteine-rich peptides in snake venom that have been developed as therapeutic agents for angiogenesis-dependent tumor growth and metastasis (Huang, 1998). Unfortunately, these molecules are very large and have low metabolic stability limiting their use for clinical applications (McLane et al., 2004, Cai and Chen, 2006). Cyclic RGD-containing pentapeptides are the most commonly used RGD-based antagonist (Ruoslahti, 1996). c(RGDf(NMe)V) a.k.a Cilengitide (EMD 121974) (Goodman et al., 2002) has effectively induced apoptosis in glioblastoma and medullablastoma (Taga et al., 2002). Integrin antagonist can be applied in combination with cytotoxic

anticancer therapy, such as chemo- or radiotherapy to maximize therapeutic efficacy. For example, Cilengitide in combination with gemcitabine inhibits highly vascularized tumor growth (Colomer, 2004; Raguse et al., 2004). It has been reported that tumor-associated endothelial cells can evade death by up-regulating $\alpha v\beta 3$ integrin upon radiation exposure. Agents such as the $\alpha v\beta 3$ inhibitor S247 have the potential to block growth of tumor cells and angiogenic vessels and cause inhibition of phosphorylation of PKB/Akt (Abdollahi et al., 2005).

Classic 2D culture conditions differ strongly from the in vivo situation and affect cell survival, proliferation, differentiation, cytoarchitecture, and migration (Kenny PA et al., 2007; Bjerkvig, 1990; Bissell, 1981; Wapita and Hay, 2002; Corcoran et al., 2003; Beliveau et al., 2010). For cancer metastasis-related studies, 3D invasion assays such as the Boyden chamber assay (trans-well migration assay) also may not properly resemble tumor cells disassociating from a solid tumor. For this purpose, cell spheroid (CS) cultures have been developed that mimic solid cancer microenvironments. This requires CS to be compact and contain an oxygen- and nutrient-depleted core, which are characteristics of solid tumors (Mueller-Klieser, 1987; Sutherlands, 1988). In addition, the ECM environment surrounding CS ideally mimics chemical (ECM protein type) and physical (rigidity, cross-linking) properties of tissue (Buxboim and Discher,2010; Friedl and Wolf, 2010, Leventhal et al., 2009). In ***chapter 6***, we describe the development a novel CS formation method in 3D collagen gels that fulfills these criteria and, for the first time, can be performed in high throughput with high accuracy and reproducibility.

There are several advantages of our approach based on microinjection over other methods. For one, we combined CS formation and gel embedding into a single step, thereby shortening preparation time from days to minutes. Secondly, CS formation from a broad spectrum of cell can be achieved without additive, e.g. matrigel such as used by others (Ivascu and Manfred, 2006, 2007). Thirdly, CS are produced with uniform size and shape with predefined spatial distribution, making this method ideal for HTS. Unlike other techniques, 2D tissue culturing steps are completely omitted, and freshly isolated tumor samples of mouse and human biopsies can be used directly for CS formation. Consequently, we have designed an automated 3D culture syste

that may be applied to drug screens for personalized treatment strategies.

According to several studies, β1 integrins support initiation and growth of breast cancer. By blocking β1 integrins with antibodies, breast tumors in mice have been sensitized to radiotherapy, indicating that β1 integrins may be suitable drug targets for breast cancer (White et al., 2004; Park et al., 2006). In ***chapter 7***, we describe that silencing β1 in breast cancer cells indeed suppresses tumor growth but can also lead to enhanced intravasation and metastasis. Our data support a model where in the absence of β1 integrins, an epithelial-to-mesenchymal (EMT) transition is induced through transcriptional down regulating of E-cadherin by an altered balance between the ZEB and mir-200 families. Consequently, cells shift from cohesive multicellular strand invasion to individual cell migration in 3D matrices. This likely enables tumor cells to intravasate more efficiently through enhanced migration by eliminating the burden to travel as collective units (dragging force) or it may up-regulate survival mechanisms to resist the sheer stress within the blood vessel; or speed up.

Experiments using inhibitory peptides and α2 subunit silencing constructs, indicate that α2β1 is the β1 integrin that acts as a metastasis suppressor in this system. Interestingly, this integrin was very recently shown to be inversely correlated with breast cancer progression (Ramirez et al., 2010). Nevertheless, we do not detect a general loss of α2 or β1 integrins to correlate with E-cadherin loss. Histological samples provide a snapshot in the dynamic process of metastasis process and may not reveal key transient malignant modifications in the metastatic cascade; especially if these are rare and transient. Indeed, the down-regulation of E-cadherin in response to decreased β1-integrin-mediated adhesion may be such a transient process that occurs in a subpopulation of cancer cells within a tumor. Such events may be missed by the pathologist but play a role in metastasis of E-cadherin positive breast cancers.

From fundamental research to clinical application, integrins have presented themselves to be highly intriguing receptors. Besides mediating cell attachment to the microenvironment, integrins organize signal transduction cascades that regulate cell biology from proliferation and survival to migration. As such, they appear to be useful biomarkers and drug targets in multiple diseases, including cancer.

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Nederlandse samenvatting en discussie

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Embryogenese, wondheling, angiogenese en kanker metastasering zijn afhankelijk van cel motiliteit en adhesie dynamiek. Dit behelst een verschuiving in integrine expressie profielen, wat mogelijk de veranderende omgeving waarin cellen zich bevinden en zich door aanpassen weergeeft. Bijvoorbeeld, kwaadaardige cellen die van primaire naar secundaire plaatsen in het lichaam migreren komen extracellulaire matrix (ECM) tegen dat verschilt van compositie in vergelijking met hun plaats van oorsprong. In **hoofdstuk 2 en 3** van dit proefschrift wordt de rol van $\alpha5\beta1$ en $\alpha v\beta3$ fibronectine (FN)-bindende integrines in cel motiliteit en adhesie dynamiek bediscussieerd. Wij hebben aangetoond dat wanneer deze integrines ectopisch tot expressie worden gebracht in de zelfde cellulaire achtergrond (GE11), opmerkelijke verschillen in cellulaire functies (cel morfologie, cel-matrix adhesie dynamiek en lokalisatie) worden geobserveerd. Cellen die $\alpha5\beta1$ integrine tot expressie brengen, hebben een contractiele fibroblast-achtige morfologie met sterk dynamische centripetaal georiënteerde cel-matrix adhesies en migreren willekeurig. In tegenstelling tot cellen die $\alpha v\beta3$ integrines tot expressie brengen, wiens cel-matrix adhesies meer statisch en verdeeld over het basale oppervlak zijn en welke zeer persistent migreren.

Wat is de reden voor deze verschillen wanneer beide integrines interacties aangaan met FN? Een mogelijke verklaring zou de interactie van signalerings en/of adapter eiwitten met specifieke residuen binnen het cytoplasmische gedeelte van integrine zijn. Echter, experimenten waarin het α- en β-gedeelte werden verwisseld hebben laten zien dat dit waarschijnlijk niet aan de orde is. Ons onderzoek suggereert dat het eerder de manier waarop integrines met FN interacties aangaan is. Wij demonstreren dat zowel $\alpha5\beta1$ als $\alpha v\beta3$ aan geïmmobiliseerd (gestrekt) FN adheren, maar dat alleen $\alpha5\beta1$ aan oplosbaar gevouwen (inactief) FN kan binden. RGD in geïmmobiliseerd (gestrekt) FN is op dusdaninge manier georiënteerd dat het toegankelijk is voor beide integrines (Altroff et al., 2004). Het vermogen van $\alpha5\beta1$ om de RGD plaats te bereiken in oplosbaar FN wordt bewerkstelligt doordat dit integrine een additionele FN bindende regio heeft (Aota, Nomizu, and Yamada, 1994; Bowditch et al., 1994; Danen et al., 1995). Wij laten zien dat het plaatsen van de CTSEQNC hypervariabele sequentie uit het I-achtig domein van $\beta1$ in de context van $\beta3$ er voor zorgt dat $\alpha v\beta3$

in die conditie ook aan oplosbaar FN kan binden. Er is gesuggereerd dat de $\alpha5\beta1$ hypervariabele sequentie bindt aan de PHSRN "synergie" regio in IIIFN9 om de interactie met de RGD regio in IIIFN10 te stabiliseren door de hellingshoek tussen IIIFN10en IIIFN9 te veranderen, en daardoor de RGD lussen vrij te leggen. Onze bevindingen demonstreren dat integrine $\alpha v\beta3$, zelfs als het door verschillende mutaties vast zit in een hoge affiniteit toestand, slecht bindt aan oplosbaar FN omdat deze functionaliteit ontbreekt (Danen et al., 1995; Sechler, Corbett, and Schwarzbauer, 1997).

De associatie met de synergie regio verklaart mogelijk het klaarblijkelijke specifieke vermogen van $\alpha5\beta1$ voor het vormen van een "houtgreep" met FN en het bewerkstelligen van adhesie versterking (Friedland, Lee, and Boettiger, 2009; Roca-Cusachs et al., 2009). Belangrijker nog, onze bevindingen wijzen er op dat het wisselen tussen deze twee integrines die dusdanig verschillende ligand-interacties vormen, een sterke invloed heeft op intracellulaire processen, met effecten op RhoA activiteit, contractiliteit van het cytoskelet en ECM opbouw.

In **hoofdstuk 4** wordt een nieuwe $\alpha v\beta3$ bindingspartner beschreven die mogelijk ook bijdraagt aan de specifieke $\alpha v\beta3$-gemedieerde effecten op cel morfologie. MacMarcks (MRP) is betrokken bij de activatie van integrines en cel spreiding door het reguleren van het corticale actine netwerk (Jin and Li, 2002). Interessant is dat de expressie van $\beta3$ integrine leidt tot verminderde expressie van MRP op transcript niveau. Wij hebben gedemonstreerd dat de regio in de nabijheid van het NITY domein van het $\beta3$ gedeelte, wanneer geassocieerd met de αv subunit, zorgt voor verminderde expressie van MRP. Niettemin leidt suppressie van MRP expressie niet tot de bevordering van cel spreiding in de oorspronkelijke cellijn, en overexpressie heeft geen effect op cel spreiding in cellen die $\alpha v\beta3$ gebruiken voor adhesie. Dit suggereert dat $\beta3$ noodzakelijk is voor MRP lokalisatie en expressie maar dat MRP niet essentieel is voor $\alpha v\beta3$-gemedieerde cel spreiding (in tegenstelling tot $\beta2$ integrines, waarvan is aangetoond dat ze MRP-afhankelijk zijn voor cel spreiding (Li et al., 1996)).

Zoals beschreven in **hoofdstuk 5**, gaan bepaalde ziektes gepaard met veranderende expressie of functionaliteit van integrines. Bijvoorbeeld, hoge expressie niveaus van verscheidende types integrines zijn in een aantal soorten kanker gecorreleerd

aan tumor progressie (Mizejewski, 1999). Om die redenen zijn antagonisten zoals peptidomimetics en monoklonale antilichamen tegen $\alpha5\beta1$ of $\alpha v\beta3$ ontwikkeld. Disintegrines zijn RGD-bevattende cysteine-rijke peptides in slangengif die verder ontwikkeld zijn als therapeutische middelen voor angiogenese-afhankelijke tumor groei en metastasering (Huang, 1998). Deze moleculen zijn echter erg groot en hebben een lage metabolische stabiliteit waardoor hun therapeutische toepassing beperkt is (McLane et al., 2004; Cai and Chen, 2006). Cyclisch RGD-bevattende pentapeptides zijn de meest gebruikte RSD-gebaseerde antagonisten (Ruoslathi, 1996). c(RGDf(NMe)V), ook wel Cilengitide genoemd (EMD 121974) (Goodman et al., 2002) induceert efficiënt apoptose in glioblastoma en medullablastoma (Taga et al., 2002). Integrine antagonisten kunnen worden toegepast in combinatie met cytotoxische antikanker therapie, zoals chemo- of radiotherapie om de therapeutische efficiënte te maximaliseren. Bijvoorbeeld, Cilengitide in combinatie met gemcitabine inhibeert tumoren met geavanceerd vasculatuur (Colomer, 2004; Raguse et al., 2004). Het is beschreven dat tumor-geassocieerde endotheel cellen kunnen ontsnappen aan cel dood door na blootstelling aan radiatie $\alpha v\beta3$ verhoogd tot expressie te brengen. Moleculen zoals de $\alpha v\beta3$ inhibitor S247 kunnen potentieel de groei van tumor cellen en angiogenese blokkeren en de fosforylering van PKB/Akt inhiberen (Abdollahi et al.m 2005).

Klassieke 2D kweek condities verschillen sterk van de in vivo situatie en beïnvloeden cel overleving, proliferatie, differentiatie, cytoarchitectuur, en migratie (Kenny PA et al., 2007; Bjerkvig, 1990; Bissell, 1981; Wapita and Hay, 2002; Corcoran et al., 2003; Beliveau et al., 2010). Voor metastase gerelateerde studies geven 3D invasie studies zoals de Boyden kamer analyse (trans-wel migratie analyse) waarschijnlijk ook niet de juiste representatie van tumor cellen die zich afscheiden van een solide tumor. Om deze reden zijn cel sferoïde (CS) culturen ontwikkeld die het micromilieu van solide tumoren nabootst. Deze CS behoren compact te zijn met een zuurstof- en voedingstoffen-loze kern. Dit is karakteristiek voor solide tumoren (Mueller-Klieser, 1987; Sutherlands, 1988). Daarbij behoort het ECM milieu dat de CS omringt dezelfde chemische (ECM eiwit type) en fysische (stijfheid, kruis-koppelingen) eigenschappen te hebben als dat van weefsel (Buxboim and Discher,2010; Friedl and Wolf, 2010, Leventhal et al., 2009). In **hoofdstuk 6** beschrijven we de ontwikkeling van een

nieuwe CS formatie methode in 3D collageen gelen die voldoet aan deze kriteria en die ook, voor het eerst, op een “high throughput” manier met hoge accuratie en reproduceerbaarheid uitgevoerd kan worden.

Onze methode, die gebaseerd is op microinjecties, levert een aantal voordelen op in vergelijking met andere methodes. Ten eerste, combineren wij CS formatie en gel inbedding in één stap. Op deze manier wordt de preparatie tijd verkort van dagen tot minuten. Ten tweede, formatie van CS uit een breed spectrum van cellen kan worden bereikt zonder additieven, zoals matrigel dat door andere groepen wordt gebruikt (Ivascu and Manfred, 2006, 2007). Ten derde, CS worden in gelijke groottes en vormen geproduceerd met een vooraf gedefinieerde ruimtelijke distributie, wat deze methode geschikt maakt voor microscopische screening. In tegenstelling tot andere technieken wordt de 2D weefselkweek stap overgeslagen en kunnen vers geïsoleerde tumor monsters uit muis en humane biopten direct gebruikt worden voor CS vorming. Met deze nieuwe methode hebben wij een geautomatiseerd 3D kweek systeem ontwikkeld dat gebruikt kan worden voor het testen van medicijnen voor gepersonaliseerde behandelings strategieën.

Volgens verschillende studies beïnvloedt β1 integrine de initiatie en groei van borstkanker. Blokkeren van β1 integrine met antilichamen maakt borstkanker tumoren in muizen gevoelig voor radiotherapie, wat aangeeft dat β1 integrine waarschijnlijk een geschikt doel is voor de ontwikkeling van medicijnen tegen borstkanker (White et al., 2004; Park et al., 2006). In **hoofdstuk 7** beschrijven we hoe het uitschakelen van β1 in borstkanker cellen inderdaad de groei van tumoren onderdrukt. Dit kan echter ook leiden tot verhoogde intravasatie en metastasering. Onze data ondersteunt een model waarin, bij de afwezigheid van β1 integrine, een epitheliale-tot-mesenchymale (EMT) transitie geïnduceerd wordt door E-cadherine op transcriptie niveau naar beneden te reguleren ten gevolge van een wisseling in de balans tussen de ZEB en mir-200 famillies. Vervolgens verschuiven de cellen van een samenhangende multicellulaire streng invasie naar migratie van individuele cellen in 3D matrices. Waarschijnlijk stelt deze verschuiving tumor cellen in staat tot een efficiëntere manier van intravasatie met een verhoogde migratie doordat de ballast van het migreren als een collectieve eenheid afvalt of doordat overlevings mechanismen om de stroming in het bloedvat

te weerstaan worden geactiveerd.

Experimenten die gebruik maken van inhiberende peptides en α2 subeenheid silencing constructen duiden aan dat van de β1 integrines de α2β1 metastasering onderdrukt in dit systeem. Van dit integrine is reeds aangetoond dat het omgekeerd gecorreleerd is met borstkanker progressie (Ramirez et al., 2010). Desondanks detecteren wij in patient materiaal geen algemeen verlies van α2 of β1 integrines dat gecorreleerd is aan E-cadherine verlies. Histologische secties geven alleen een momentopname uit het dynamische process van metastasering en missen mogelijk een belangrijke kwaadaardige modificatie in de metastase cascade. De verminderde expressie van E-cadherine naar aanleiding van de vermindering van β1-integrine-gemedieerde adhesie is mogelijk een tijdelijk proces dat in een subpopulatie van kanker cellen in een tumor voorkomt. Dit soort evenementen kunnen makkelijk over het hoofd worden gezien door de patholoog terwijl ze een belangrijke rol spelen in de metastasering van E-cadherine positieve borstkanker.

Van fundamenteel onderzoek tot klinische toepassingen blijken integrines hoogst interessante receptoren. Naast het mediëren van cel adhesie aan de micro-omgeving induceren integrines ook signaal transductie cascades die proliferatie, overleving en migratie reguleren. Zodoende blijken integrines bruikbare biomarkers en kunnen ze worden gebruikt als doel om medicijnen tegen te ontwikkelen in verschillende ziektes, waaronder kanker.

Curriculum Vitae (CV)

Hoa Hoang Truong was born on the 3rd of November 1977 in Reno, Nevada, U.S.A. In 1996, she completed her high school education at Edward C. Reed High school in Sparks, Nevada. During her Bachelor study, she interned at the Pharmacology department of University of Nevada, Reno (UNR) under the supervision of Dr. Svetlana Todorova, PhD, MD. Soon after, she obtained a Bachelor of Science degree in Biology and a minor in chemistry and computer science, in 2001 from UNR. After graduation, she worked for Sierra Biomedical, a division of Charles Rivers Laboratories as formulation technician. Following a brief career in the pharmaceutical industry, she returned to academia to pursue a Master of Science program, Molecular Cell Biology and Bioinformatics, at the University of Amsterdam in 2003. In 2004, she performed her Master internship in the Lab of Dr. Arnoud Sonnenberg, PhD under the supervision of Dr. Iman van den Bout, PhD and received her Master degree from the University of Amsterdam in 2006. In the same year, she started her doctoral study in the lab of Prof. Dr. Bob van de Water, PhD in the division of Toxicology, LACDR, Leiden University in the Group of Dr. Erik Danen, PhD. Currently, she is a post-doctoral researcher at the department of Molecular Biophysics at the Utrecht University in the Group of Prof. Dr. Hans Gerritsen, PhD.

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