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ANCHOR OR ACCELERATE – A STUDY ON CANCER CELL ADHESION AND MOTILITY

by

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To my dear family

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Anchor or Accelerate – A Study on Cancer Cell Adhesion and Motility
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ABSTRACT

Cell migration and adhesion to the extracellular matrix (ECM) are crucial in many biological and pathological processes such as morphogenesis, tissue repair, inflammatory responses, survival, and cancer. Cell-matrix adhesion is mediated by the integrin family of transmembrane receptors, which not only anchor cells to their surroundings, but also transmit bidirectional signalling at the cell surface and couple the ECM to the cytoskeleton. Another group of adhesion receptors are the syndecan proteoglycans, which engage the ECM and possess signalling activity in response to a variety of ligands. Cell migration is a complex process that requires spatial and temporal coordination of adhesion, cell contractility, intracellular traffic of integrins, and matrix turnover by matrix metalloproteinases (MMPs). Thus, integrins and syndecans, as well as MMPs, play essential roles in cancer cell migration and invasion. The understanding of the cooperation of syndecans and integrins was broadened in this thesis study. The results reveal that syndecan-1 functions in concert with α2β1 integrin in cell adhesion to collagen, whereas syndecan-4 is essential in α2β1 integrin-mediated matrix contraction. Finally, oncogenic K-Ras was shown to regulate α2β1 integrin, membrane-type 1 MMP, and syndecan-1 and -4 expression and their cooperation in cell invasion.

Epithelial-mesenchymal transition (EMT) is fundamental during embryogenesis and organ development. Activation of EMT processes, including the upregulation of mesenchymal intermediate filament protein vimentin, has also been implicated in the acquisition of a malignant phenotype by epithelial cancer cells. Members of the protein kinase C (PKC) superfamily are involved in cell migration and various integrin-dependent cellular functions. One aim of this work was to shed light on the role of vimentin in the regulation of integrin traffic and cell motility. In addition, the mechanism by which vimentin participates in EMT was investigated. The results show that integrin recycling and motility are dependent on the PKCε–mediated phosphorylation of vimentin. In addition, vimentin was found to be a positive regulator of EMT and regulate the expression of several migratory genes. Specifically, vimentin governs the expression of receptor tyrosine kinase Axl, which is implicated in tumour growth and metastasis.

Taken together, the findings described in this thesis reveal novel aspects of the complex interplay between distinct cellular components: integrins, syndecans, and the vimentin cytoskeleton, which all contribute to the regulation of human cancer cell adhesion, migration, and invasion.

Keywords: Integrin, syndecan, vimentin, Axl, adhesion, migration

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TIIVISTELMÄ

Solun tarttuminen soluväliaineeseen eli sidekudosmatriksiin ja siellä liikkuminen ovat elintärkeitä tapahtumia monissa biologisissa ja patologisissa prosesseissa kuten alkionkehityksessä, kudosten paranemisessa, tulehdukseen liittyvissä reaktioissa ja syövässä. Integriinien transmembraanisen reseptoriperheen avulla solut tarttuvat ympäristöönsä. Integriinit myös välittävät viestejä solun ja sen ympäristön välillä ja liittävät soluväliaineen solun tukirankaan. Syndekaani-proteoglykaanit ovat toinen tarttumisreseptoriperhe, joka sitoutuu soluväliaineeseen ja välittää erilaisten reseptoriin sitoutuvien ligandien toimittamia viestejä. Solun liikkuminen on monimutkainen tapahtumasarja, jossa mm. solun tarttuminen, supistuminen, integriinien solunsisäinen liikenne ja matriksin metalloproteinaasien (MMP:ien) huolehtima soluväliaineen muokkaaminen yhdistyvät tarkassa järjestyksessä. Niin integriinit ja syndekaanit kuin MMP:tkin ovat välttämättömiä syöpäsolun liikkumisessa ja invaasiossa. Tässä väitöskirjatutkimuksessa selvitettiin integriinien ja syndekaanien välisiä vuorovaikutuksia. Tulokset osoittavat, että syndekaani-1 toimii yhdessä $\alpha 2\beta 1$-integriinin kanssa solun tarttumisessa kollageeniin, kun taas syndekaani-4 on keskeinen $\alpha 2\beta 1$-integriinivälitteisessä sidekudoksen supistumisessa. Lopuksi osoitettiin, että K-Ras-syöpägeeni säätelee $\alpha 2\beta 1$-integriinin, membraanityyppi-1-MMP:n ja syndekaani-1 ja -4:n ilmentymistä sekä yhteistyötä solun invaasiossa.

Epiteeli-mesenkyymimuutos (EMT) on perustavanlaatuinen tapahtuma alkionkehityksessä ja elinten muodostumisessa. EMT-prosessien aktivoitumista, mukaanlukien välikokoisen säieproteiini vimentiinin ilmentymistä, tarvitaan myös epiteelisyöpäsolujen muuttumisessa pahanlaatuisiksi. Proteiinikinaasi C (PKC)-perhe liittyy solun liikkumiseen ja moniin integriinivälitteisiin solun toimintoihin. Yksi tämän tutkimuksen tavoitteista oli selvittää vimentiinin osuus integriiniliikenteessä, solun liikkumisessa ja EMT-prosessissa. Tulokset osoittavat, että PKCε-välitteistä vimentiinin fosforylaatiota tarvitaan integriinin kierrättämisessä ja solun liikkumisessa. Vimentiinin havaittiin käynnistävän solussa EMT-tapahtumasarjan ja säätelevän useiden solun liikkumiseen vaikuttavien geenien ilmentymistä. Lopulta todistettiin, että vimentiini määrää syöpäkasvaimen kasvuun ja metastaasiin osallistuvan reseptori-tyrosiinikinaasi Axl:n ilmentymisestä.

Tässä väitöskirjatyössä selvitettiin integriinien, syndekaanien ja vimentiinitukirangan monimutkaisia vuorovaikutuksia. Löydökset tuovat uudenlaista tietoa syöpäsolujen tarttumisen, liikkumisen ja invaasion säätelystä.

Avainsanat: Integriini, syndekaani, vimentiini, Axl, adheesio, migraatio

TABLE OF CONTENTS

ABBREVIATIONS

2D, 3D	two-dimensional, three-dimensional
ADP	adenosine diphosphate
AP2	adaptor protein 2
Arf	ADP-ribosylation factor
α-SMA	α-smooth muscle actin
aPKC	atypical protein kinase C
ATP	adenosine triphosphate
BIM	bisindolylmaleimide
C1-TEN	C1 domain-containing phosphatase and TENsin homologue
Cdk	cyclin-dependent kinase
CHO	Chinese hamster ovary
CML	chronic myelogenous leukaemia
COX-2	cyclo-oxygenase-2
CS	chondroitin sulphate
Dab2	Disabled-2
DAG	diacylglycerol
DLC1	deleted in liver cancer 1
DRF	diaphanous-related formin
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-to-mesenchymal transition
ERK	extracellular signal-regulated kinase
ERM	ezrin-radixin-moesin
EV1	human echovirus 1
F-actin	filamentous actin
FAK	focal adhesion kinase
FBS	fetal bovine serum
FERM	4.1 ezrin radixin moesin
FGF	fibroblast growth factor
FN	fibronectin
GAG	glycosaminoglycan
GAP	GTPase activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
Gas6	growth arrest-specific gene 6
GDI	guanine nucleotide dissociation inhibitor
GEF	guanosine nucleotide exchange factor
GFR	growth factor receptor
GIST	gastrointestinal stromal tumours
GPI	glycosylphosphatidylinositol
Grb2	growth factor receptor-bound protein 2
GDP	guanosine diphosphate

GTP	guanosine triphosphate
HGF	hepatocyte growth factor
HS	heparin sulphate
HSPG	heparin sulphate proteoglycan
ICD	intracellular domain
IF	intermediate filament
Ig	immunoglobulin
IL	interleukin
IL-15Rα	IL-15 receptor α
ILK	integrin-linked kinase
JNK	c-Jun N-terminal kinase
KLF8	Krüppel-like factor 8
Lck	leukocyte-specific protein tyrosine kinase
LEF-1	lymphoid enhancer-binding factor 1
LPA	lysophosphatidic acid
mDia1	mammalian diaphanous 1
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblast
MET	mesenchymal-epithelial transition
MHCIIA/B	myosin heavy chain type II A/B
MIDAS	metal-ion dependent adhesion site
MLC	myosin II light chain
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MT-MMP	membrane-type MMP
NF-κB	nuclear factor κ-light-chain enhancer of activated B cells
PAK	p21-activated kinase
PAR	partitioning-defective
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDZ	post-synaptic density 95; disc large tumour suppressor; zonula occludens 1
PG	proteoglycan
PI	phosphatidyl inositol
PI3K	phosphatidyl inositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PKCεRE	PKCε reconstituted
PLC	phospho lipase C
PLK1	Polo-like kinase 1
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homologue
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
ROCK	Rho kinase

RTK	receptor tyrosine kinase
RT-PCR	reverse transcription PCR
Sdc	syndecan
SH2	phosphotyrosine-binding Src homology 2
siRNA	small-interfering RNA
STAT3	signal transducer and activator of transcription 3
TAM	Tyro3, Axl, Mer
TGF-β	transforming growth factor-β
Tiam1	T-lymphoma invasion and metastasis inducing protein 1
TIMP	tissue inhibitor of metalloproteinases
VAM	vimentin-associated matrix adhesion
VASP	vasodilator-stimulated phosphoprotein
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
wt	wild-type
ZEB	zinc-finger E-box binding homeobox
ZO-1	zonula occludens 1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals I-IV:

I Ivaska J, Vuoriluoto K, Huovinen T, Izawa I, Inagaki M, Parker PJ.
PKCepsilon mediated phosphorylation of vimentin controls integrin recycling and motility.
EMBO J. 2005 Nov 16;24(22):3834-45.

II Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, Tiron C, Lorens JB, Ivaska J.
Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer.
Oncogene. 2010 Nov 8. [Epub ahead of print]

III Vuoriluoto K, Jokinen J, Kallio K, Salmivirta M, Heino J, Ivaska J.
Syndecan-1 supports integrin α2β1-mediated adhesion to collagen.
Exp Cell Res. 2008 Nov 1;314(18):3369-81. Epub 2008 Jul 15.

IV Vuoriluoto K, Högnäs G, Meller P, Lehti K, Ivaska J.
Oncogenic K-ras regulates α2β1-integrin, MT1-MMP, syndecan-1 and -4 expression and their interplay in collagen matrix contraction and cell invasion into collagen.
Matrix Biology. Under revision.

In addition, some unpublished data are presented in this thesis.

1. INTRODUCTION

The leading cause of death worldwide is cancer (malignant tumour, neoplasm). Cancer arises from extreme phenotypical and biochemical changes in one single cell. Cancer becomes a life-threatening disease when the cell proliferates and the resulting cells acquire properties of invasion into adjacent tissues, as well as the ability to spread into distant organs, a process referred to as metastasis. In order to begin the multistep process of metastasis, most cancer cells need to activate epithelial-mesenchymal transition (EMT) and many signalling pathways (Kalluri and Weinberg, 2009). Three major components form the cytoskeleton: actin filaments, intermediate filament (IF) proteins, and microtubules. Vimentin is the main IF protein of the mesenchymal cell cytoskeleton. It is also expressed in certain adult tissues and during developmental processes. In addition, vimentin has been associated with a motile and invasive phenotype, functioning in the organisation of proteins critical for cell adhesion, migration, and signalling. Thus, vimentin is commonly used by EMT researchers as a marker of mesenchymal cells (Ivaska *et al.*, 2007).

Integrins are transmembrane, heterodimeric adhesion receptors that have fundamental tasks in cell signalling across the plasma membrane, in addition to their major role in adhesion. Briefly, integrins play key roles in development, immune responses, hemostasis, and cancer (Hynes, 2002). Syndecan proteoglycan transmembrane receptors interact with many cytokines, chemokines and matrix metalloproteinases (MMPs), as well as extracellular matrix (ECM) proteins, through their extracellular glycosaminoglycan chains. The short cytoplasmic domains of syndecans and integrins have no intrinsic kinase activity; rather, they interact with cytoskeletal proteins. In summary, the cell-surface receptor families integrins and syndecans are linked to one another and even cooperate during adhesion and migration (Couchman, 2010).

Migration is a multistep process that leads to the translocation of cells on or through tissue. Put simply, it is a function of regulated adhesion and deadhesion. For some cell types, cell migration is fundamental for their function after morphogenesis and terminal differentiation. Most cells become motile only for regeneration and neoplastic processes. The role of matrix metalloproteinases is somewhat contradictory; the proteolytic remodelling of the ECM is fundamentally important for many physiological events, but it is also essential in cancer cell invasion and metastasis (Friedl and Wolf, 2009). The major proteinase necessary for invasion is membrane-type 1 MMP (MT1-MMP) (Sabeh *et al.*, 2004).

This thesis explores the role of vimentin in integrin traffic, cell motility, and EMT. Specifically, the contribution of vimentin to the regulation of Axl receptor tyrosine kinase is elucidated. In addition, the interactions of $\alpha 2\beta 1$ integrin and syndecan proteoglycans on the major ECM component collagen in relation to cell adhesion, actin organisation, integrin signalling, matrix contraction, and invasion are described.

2. REVIEW OF THE LITERATURE

2.1. CELL ADHESION

The ECM not only supports tissue structure and anchors the cells, but also participates in the regulation of cell behaviours, specifically morphology, metabolism, differentiation, and survival. The complex meshwork of ECM comprises various proteins and polysaccharides like fibronectin (FN), laminin, proteoglycans secreted by cells, and collagen, which is the predominant ECM protein in the body (Hynes, 2009). Important in determining the physical tissue's properties, matrix proteins are assembled into fibrils and networks. Collagen fibrillogenesis *in vivo* is dependent on integrins and other ECM components, and follows FN matrix assembly. FN embodies a collagen-binding region, and fibres of the direct binding partners collagen and FN often colocalise in the ECM (Velling *et al.*, 2002).

Adhesion links cells to the neighboring cells, and to the surrounding matrix. Through adhesion, cells constitute distinct tissues and organs in vertebrates (Heino and Kapyla, 2009). Proteins participating in adhesion influence basically all cellular processes, including morphogenesis, differentiation, migration, proliferation, and survival. The transmembrane integrin receptor family members mediate adhesion to the ECM and trigger intracellular signalling pathways (Zaidel-Bar and Geiger, 2010). Integrins are the main but not the only receptors mediating adhesion. A multiplicity of adhesion receptors and molecules exist: cadherins, selectins, immunoglobulins, glycoproteins, and many more. For example, the contribution and adhesion mechanisms of the cell surface receptor CD44 have not been intensively studied. CD44 binds preferentially to carbohydrate polymers and other surface receptors, including hyaluronic acid, syndecan proteoglycans, and receptor tyrosine kinases (RTKs) (Schmidt and Friedl, 2010).

Cell-ECM adhesion sites constitute a variety of different kinds of structures, all containing transmembrane integrin family proteins and cytoplasmic actin. The adhesion sites can be classified into focal complexes, classical focal adhesions, and fibrillar adhesions according to size, intracellular localisation, molecular composition, and the morphology of the formation. Dot-like focal complexes mature into strong focal adhesions under tension. Focal adhesions connect to actin stress fibres, whereas fibrillar adhesions associate to thin actin filaments and assemble FN fibrils. Typically, fibrillar adhesions contain tensin and $\alpha 5\beta 1$ integrin but no vinculin. Contractile forces are dispensable for fibrillar adhesion formation but not for focal complexes or focal adhesions (Hinz *et al.*, 2003). The complex cell-ECM connection sites, focal adhesions, are connections between integrins and the cytoskeleton. They are highly dynamic, multi-protein sites of cell-matrix adhesion through which cytoskeletal motion is transmitted. Focal adhesions are functional units consisting of integrin clusters with aggregations of cytoskeletal structural linker/adaptor proteins like talin, α-actinin, kindlins, vinculin, and the Rho family of cytoskeletal regulatory G proteins. In focal

adhesions, possibly >100 proteins knit the cell cytoskeleton to the plasma membrane, and finally to the surrounding ECM. Talin is acknowledged as a connector that links integrins to the actin cytoskeleton and regulates integrin activity by altering integrin ligand affinity. α-actinin modulates the strength and stability of the linkage; actin-binding adaptor vinculin and integrin-linked kinase (ILK) maturate and stabilise focal adhesions. Focal adhesions are regulated by phosphorylation and the binding of various additional signalling partners, including Src kinase, focal adhesion kinase (FAK), the multi-domain scaffold protein paxillin, protein kinase Cα (PKCα), and kindlins (Legate *et al.*, 2009). Kindlins are required for the correct assembly of cell-ECM adhesions where they directly bind to β integrin tails (Figure 1) (Kloeker *et al.*, 2004).

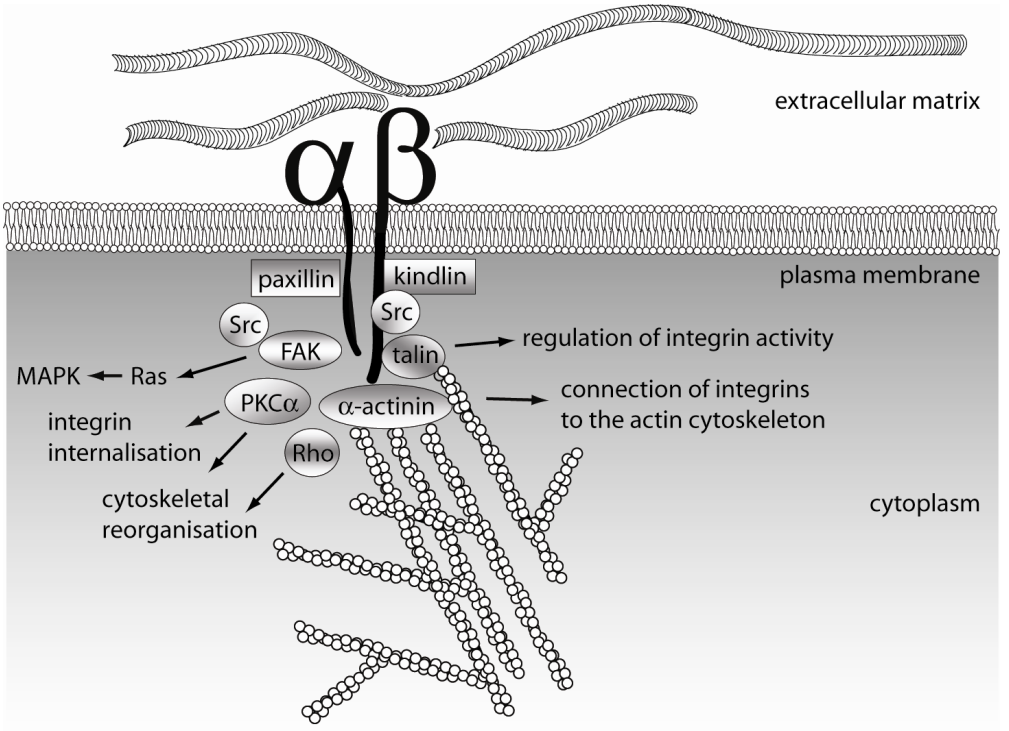

Figure 1. Focal adhesion signalling. Integrin clusters connect the ECM and the cytoskeleton to each other in focal adhesions. Dynamic focal adhesion signalling plays essential roles in a multiformity of cellular functions. The figure illustrates an αβ integrin heterodimer unit at the plasma membrane engaged by ECM ligands. Intracellular adaptors link the integrin cytoplasmic tail to actin fibres and activate downstream signalling cascades.

Kinases are enzymes that transfer phosphate groups from high-energy molecules to their substrates. Thus, kinases induce protein phosphorylation in focal adhesions and subsequently transduce extracellular signals in the intracellular pathways. The non-receptor cytoplasmic tyrosine kinase Src was the first oncogene identified (Levinson *et al.*, 1978). In addition to its role in tumourigenesis, Src is indispensable in a wide range

of normal physiological pathways (Martin, 2001). Plasma membrane receptors, including integrins and RTKs, activate Src. Under normal conditions, Src plays a role in maintaining cell homeostasis, as well as in countless physiological functions, including cell proliferation and survival, regulation of cell shape and cytoskeleton, cell-cell and cell-matrix adhesion dynamics, invasion, and cell motility (Guarino, 2010). During ECM engagement, Src directly interacts with the β3 integrin cytoplasmic tail (Huveneers *et al.*, 2007) and phosphorylates β1 integrin, subsequently suppressing cytoskeletal contractility (Huveneers *et al.*, 2008). Cell adhesion by integrin receptors results in FAK autophosphorylation and activation. Tyrosine phosphorylation of FAK allows for Src binding and the activation of Src in the forming FAK-Src complex. The activated Src further phosphorylates FAK and creates new protein binding sites, amplifying the kinase activity of FAK. The highly active FAK-Src complex promotes the phosphorylation of many Src substrates, such as paxillin, and activates several pathways leading, for example, to the formation of membrane protrusions. In migrating cells, the FAK-Src complex eventually coordinates focal adhesion dynamics at the leading edge and at the rear (Figure 1) (Huveneers and Danen, 2009).

Cell-cell connections are essential in maintaining cell structure and tissue organisation. These bonds are regulated by for example cell polarity pathways. Different types of intercellular connection sites include tight junctions, adherens junctions, gap junctions, and desmosomes. Tight junctions are located at the apical-basal border of polarised epithelial cells close to the tissue lumen, and prevent the free flow of molecules and ions through the space between cells. Adherens junctions are found basal to tight junctions. Both adherens junctions and desmosomes connect epithelial cells to each other. Adherens junctions constitute of cadherin transmembrane receptors connecting to cytoskeletal actin filaments through catenins whereas in desmosomes proteins of the cadherin family attach to keratin IFs via linker proteins similar to catenins. Gap junctions are intercellular tunnels that permit the passage of small molecules and ions. They are constructed of transmembrane protein connexins (Feigin and Muthuswamy, 2009). Many of the signalling proteins recruited to these cell-cell contacts are the same as in cell-matrix connections, stabilising interactions to the actin cytoskeleton and the IFs (Jamora and Fuchs, 2002).

2.1.1. Integrins

In humans, members of the integrin receptor family play key roles in many processes such as embryonic development, immune responses, cancer, and other pathogenic conditions. These receptors are expressed in all metazoan cell types, except erythrocytes. The number of different integrin heterodimers expressed in an organism correlates with the complexity of the organism. Integrins are heterodimeric cell surface receptors that mediate binding and bidirectional signalling between cells and the ECM. Integrins constitute of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic tail. The expression and combination of 18 α and 8 β subunits determine ligand specificity since heterodimers are substrate specific, though

multivalent (Figure 2). Following substrate binding and adhesion, integrins trigger signal transduction pathways and modulate cellular behaviour orchestrating cell motility, polarity, proliferation, ECM assembly, and survival. The activated downstream signalling pathways are regulated in many ways: the assembly of the modules, the plasma membrane order, and endocytosis (Hynes, 2002).

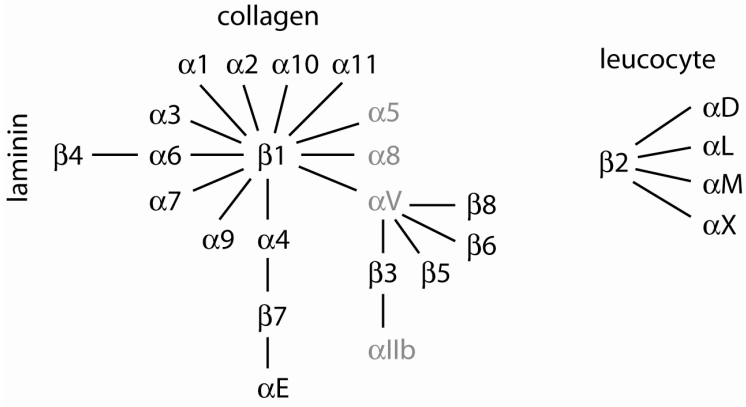

Figure 2. The integrin receptor family. The α and β subunits that couple to form heterodimers are connected by a line. The collagen-specific integrin receptors comprise heterodimers with α subunit partners α1, α2, α10, and α11; the laminin-specific α3, α6, and α7; the leucocyte-specific αD, αL, αM, and αX; the arginine-glycine-aspartic acid (RGD)-recognising are those in grey: α5, α8, αV, and αIIb. The data was collected from (Hynes, 2002) and (Chen and Sheppard, 2007).

Integrins are the major receptor group mediating cell-matrix adhesion. Many cellular responses depend on anchorage by integrins. After ligand engagement, integrins multimerise in clusters at the plasma membrane, connect to the cytoskeleton, and stimulate intracellular signalling pathways (Schmidt and Friedl, 2010). There are four cellular collagen-specific integrin receptors in vertebrates: α1β1, α2β1, α10β1, and α11β1. Each of the collagen-binding integrin α subunits contains an αI domain, which embodies the main collagen-binding site. This metal ion-dependent adhesion site (MIDAS) holds a coordinating divalent cation, Mg^{2+} or Mn^{2+} ion, required for high affinity binding to collagens (Emsley *et al.*, 2000; Valdramidou *et al.*, 2008). The structures of αI domains of collagen-binding integrins are very similar. The recognition site applicable for αI domain in collagen-binding integrins is the triple-helical sequence GFOGER (Knight *et al.*, 2000). However, there are differences in the ligand-binding mechanisms and collagen subtype specificity as well as in biological functions of different collagen-binding integrins (White *et al.*, 2004). FN- and vitronectin-binding integrins recognise the arginine-glycine-aspartic acid (RGD) motif, whose conformation in the individual ECM proteins determines specificity (Ruoslahti and Pierschbacher, 1987). Integrins lack intrinsic enzymatic activity and need to recruit signalling molecules to initiate signalling. Occupation of the extracellular ligand binding site or engagement of the cytoplasmic domains activates the integrin. The activation is characterised by conformational changes, extension and cytoplasmic tail

separation, in integrin receptor structure, which allow for increased affinity for ECM ligands (Figure 3) (Boerner *et al.*, 1996; Mitra *et al.*, 2005; Caswell *et al.*, 2009). Active integrin heterodimers gather to lipid raft focal adhesion sites together with other signalling molecules and cluster into oligomers. Myosin II exerts tension on actin cytoskeleton to mediate clustering of the ligand bound integrins, which sums the weak interactions into tight complexes (Bloor and Kiehart, 2001). The influence of integrin clustering on ligand binding is a target of fierce debate, but clustering alongside with conformational changes in integrin receptors undoubtedly effects various integrin functions such as ECM ligand binding-triggered signalling, integrin recycling, and mechanotransduction by integrin-containing adhesion sites (Shattil *et al.*, 2010).

Integrins exhibit different expression patterns in tissues. α1β1 integrin is expressed on cells of mesenchymal origin including fibroblasts, smooth muscle cells, and microvascular endothelial cells (Duband *et al.*, 1992). α2β1 integrin is an epithelial cell receptor. It is also expressed in endothelial cells, fibroblasts, and cells of hematopoietic origin (Wu and Santoro, 1994). α10β1 and α11β1 integrins are differentially distributed compared to the expression pattern of α1β1 and α2β1 integrin. Expression of both α10β1 and α11β1 integrin seems to be restricted to mesenchymal cells (Tiger *et al.*, 2001; Camper *et al.*, 2001). Knockout mice reveal distinct roles for integrins as integrins play many important roles during development and growth. Consequently, the knockout phenotypes range from developmental defects to perinatal lethality. Nevertheless, genetic compensation appears to upregulate substitutive receptor systems for replacement of the loss of some integrin functions. It should be noted that embryonic knockout and blockade of integrin function in adult animals do not necessarily produce the same phenotype and the reasons for this remain unclear and a source of controversy, but certainly one explanation is that other integrin receptors may compensate. Several integrin subunits can form different heterodimers with multiple subunits, further complicating the interpretation of knockout studies (Chen and Sheppard, 2007). β1 integrin subunit associates with 12 different α subunits to form heterodimers. Thus, β1 integrin knockout in mice generates the most severe phenotype. β1 integrin null embryos die early, and only empty implantation sites can be detected at embryonic day E5.5 (Fassler and Meyer, 1995; Stephens *et al.*, 1995). Also, α5 integrin knockout in mice is embryonically lethal (Watt and Hodivala, 1994). Conversely, the α2β1 integrin knockout mice appear substantially healthy despite the widespread expression pattern of α2β1. These mice develop and reproduce normally, but show impaired branching morphogenesis in the mammary gland and defective hemostasis due to defective platelet-collagen interactions (Chen and Sheppard, 2007; Holtkotter *et al.*, 2002).

2.1.1.1. Bidirectional integrin signalling

Integrins interact with polymerised, filamentous actin (F-actin) and focal adhesion proteins. In protrusions like lamellipodia or filopodia, integrins maintain cell shape in a manner that allows for spreading, migration, and growth. Usually, integrins are expressed on the cell surface in inactive conformation. Temporal and spatial regulation

of integrin activity is essential for proper function. Ligand binding to the extracellular domain results in integrin outside-in signalling: integrin conformational change and activation of intracellular signal transduction pathways. Control of integrin function from within the cell is referred as inside-out signalling. During inside-out (intracellular to extracellular) signalling, integrins transmit mechanical and biochemical signals from the cell interior to the exterior opposite to outside-in signalling following the binding of an intracellular signalling molecule and the consequential conformational change in the integrin structure (Figure 3). Integrin inside-out signalling is required for example for platelet activation, leukocyte migration, and coordination of adhesion during cell migration. Especially for immune cells and platelets, staying inactive until triggered is important. In platelets, αIIbβ3 integrin activation leads to accelerated blood clotting (Loftus and Liddington, 1997; Savage *et al.*, 1998). Tyrosine phosphorylations following integrin activation lead to focal adhesion assembly and activation of other kinases associated with adhesion. Integrin-activating proteins talin and kindlins can bind to the β integrin tail and induce receptor activation and subsequent downstream effects. Both talin and kindlins contain a four point one ezrin radixin moesin (FERM) domain suggested to be essential in the binding of talin to β integrin (Caswell *et al.*, 2009; Moser *et al.*, 2009; Harburger *et al.*, 2009).

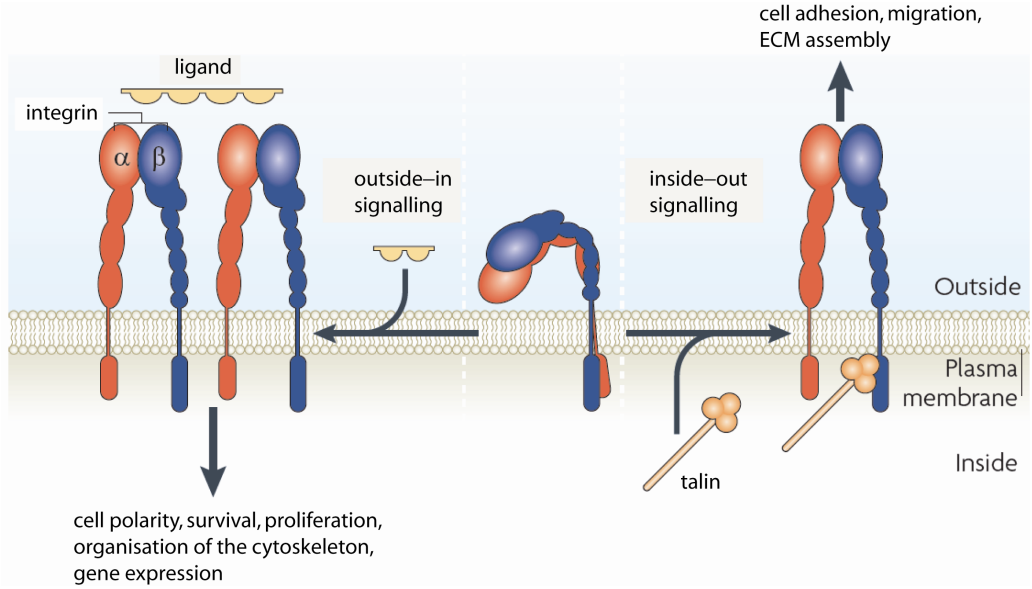

Figure 3. Integrin activation and bidirectional signalling. Upon activation either by binding of an ECM ligand (outside-in signalling) or an intracellular signalling molecule (inside-out signalling), integrins exhibit conformational changes in their structure. These include extension of the bent inactive conformation and tail separation. Adapted from (Shattil *et al.*, 2010).

Another integrin outside-in signalling mechanism besides ligand binding-triggered pathway activation is to influence the manner how growth factor receptors (GFRs) respond to their ligands (Caswell *et al.*, 2009). Integrins enable growth factor

signalling in many cases and cooperate with GFRs at multiple levels such as sequestering and activating growth factors and regulating transcriptional programs. Growth factor stimulation and integrin-mediated adhesion together support the activation of an important signalling molecule: mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK). The ERK pathway is commonly disturbed in cancers. In response to collagen binding and subsequent receptor activation, integrin α2β1 specifically activates p38α MAPK (Ivaska *et al.*, 1999). Also, FAK phosphorylation is regulated by both integrins and GFRs. Furthermore, the activated adhesion complex consisting of integrins and GFRs induces the Ras-MAPK downstream pathway shown to be responsible for the malignant phenotype (Schwartz and Ginsberg, 2002).

2.1.1.2. Integrins support cell migration through adhesion

Cell adhesion and migration are central to many biological and pathological processes including tissue repair, embryogenesis, inflammatory responses, and cancer. For migration, spatial and temporal integration of adhesion among different processes is essential. The steps of a constantly repeated migratory cycle consist of extension of protrusions driven by actin polymerisation, formation of stable attachments near the leading edge, forward movement of the cell body, and eventually, release of adhesions and retraction at the cell rear (Webb *et al.*, 2002). The functions of integrins in cell motility and adhesion have been studied extensively. In summary, in moving, polarised cells, integrins have a role in the formation of protrusions and new adhesion sites at the leading edge, whereas at the rear, integrins are either degraded or recycled back to the cell front (Ivaska *et al.*, 2005).

Cells use different strategies to migrate, depending on the strength and stability of adhesion. In amoeboid migration, cell shape is typically roundish to ellipsoid, and the cells embody cortical actin cytoskeleton that lacks stress fibres as well as mature focal adhesions. Mesenchymal migration used by fibroblasts, myoblasts, and many cancer cells is characterised by elongated, spindle-shaped morphology and focalised adhesion sites with substantial adhesion and traction force. Force can be generated via pulling or propulsion, and cells can glide in the matrix individually (through amoeboid or mesenchymal modes) or collectively within multicellular formations, depending on purpose. Single-cell migration applies for long-distance travelling and tissue integration during processes such as immune cell trafficking and cancer metastasis to distant sites. During cancer invasion and wound healing, the cells connected to each other by cadherins and other mechanisms migrate collectively as monolayer sheets or 3D sprouts, strands, or clusters. Complex tissues such as epithelia, ducts, glands, and vessels are built, shaped, and remodelled by cell migration through the collective mode. Collective cell migration also participates in cancer progression by local invasion. Lymphocytes, dendritic cells, and lymphoma cells with low or no traction force mediated by integrins prefer the amoeboid migration mode. They form instable, diffusely organised adhesion sites that are rapidly disassembled. Characteristics of both tissue environment and cell behaviour determine the migration mode. ECM

determinants include dimension (2D or 3D), density and gap size, stiffness, and orientation of fibres. To modulate migration, these parameters are integrated with the input from cell-cell and cell-matrix adhesion, cytoskeletal polarity and stiffness, and pericellular proteolysis (Schmidt and Friedl, 2010; Friedl and Wolf, 2010).

Integrins, as adhesion receptors, support cell migration across two-dimensional (2D) surfaces whereas migration in 3D tissue requires both integrin-dependent and -independent interactions (Schmidt and Friedl, 2010). Focal adhesion association strength correlates with cell migration and the rate of cell migration depends on integrin detachment from the ECM (Hendey *et al.*, 1992). Weaker connections to integrins allow for detachment of actin that permits cell movement, while strong adhesions transmit the force of pulling to the ECM (Hu *et al.*, 2007). Continuous, selectively regulated turnover of adhesion sites is a mechanism of regulating cell motility and reorganisation of the actin cytoskeleton. The highest velocities of integrin-dependent migration in 2D ECM substrates are reached when the adhesion strength is on intermediate level, allowing for rapid formation of new focal contacts and generation of intracellular traction forces. Efficacious adhesion to the substrate immobilises and anchores the cell. Also, proper adhesion delays retraction of the cell rear and thus slows down cell migration. However, under too gentle attachment, no mechanical forces at the leading edge are generated and the cell cannot move (Friedl and Brocker, 2000).

Three fundamental types of cellular filamentous compartments connect polymerised actin to the ECM in migrating cells. These subcompartments include sheet-like lamellipodia, cell surface projections called filopodia, and contractile actin-myosin filament bundles called stress fibres. Lamellipodia located in the cell front comprise a crosslinked meshwork of actin bundles; filopodia protrude the matrix in the leading edge and consist of linear and parallel actin filament bundles (Nobes and Hall, 1999; Small *et al.*, 1996). The family of guanosine triphosphate (GTP) hydrolysing enzymes, the small GTPases, also called the Ras superfamily, regulate actin dynamics and operate as molecular switches for several cellular signalling events. The Ras superfamily is further divided into several subfamilies. To mention a few, the Ras subfamily is responsible for cell proliferation, the Rho subfamily for cytoskeletal dynamics and morphology, the Rab subfamily for membrane traffic, and the ADP-ribosylation factor (Arf) subfamily for vesicle transport. The small GTPases are activated by a nucleotide change from guanosine diphosphate (GDP) to GTP, a reaction catalysed by guanine exchange factors (GEFs). In nonadherent cells, unactivated Rho remains cytoplasmic in a complex with Rho guanine nucleotide dissociation inhibitor (GDI). GTPase-activating proteins (GAPs) initiate the reversion of the cycle by binding the GTPase to GDP and thus switching it off (Figure 4). The activity of GEFs and GAPs and their recruitment to adhesions are regulated by integrin signalling (Wiesner *et al.*, 2005).

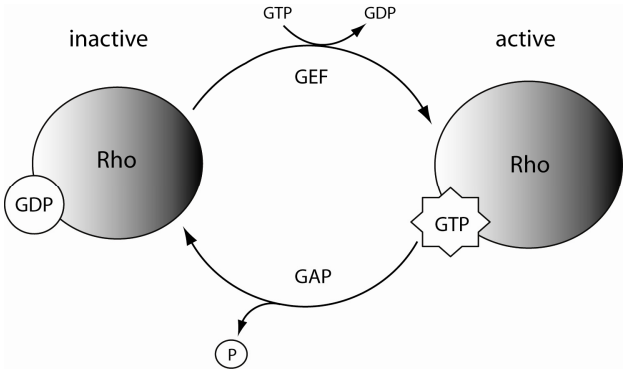

Figure 4. Regulation of the Rho GTPase activity. A schematic drawing of the GDP-GTP exchange cycle regulating the activity of small GTPases. inhibiting The nucleotide change from GDP to GTP is triggered by specific GEFs; the GAPs reverse the reaction. GTPase-activating protein; GEF, guanine exchange factor.

The Rho family GTPases regulate all the steps of migration from the formation of new adhesions at the cell front to the disassembly and retraction at the cell rear. During migration, small, nascent focal adhesions are formed at the cell front. They either mature to focal adhesions or get rapidly disassembled depending on myosin contractility regulated by Rho (Webb *et al.*, 2002). In focal adhesions, the cycle of GTPase activation regulates actin dynamics and thus cell polarity and migration, for which the most important GTPases are the Rho family proteins RhoA, Rac1, and Cdc42. The Rho family GTPase activation level depends on the repertoire of integrin receptors expressed on a cell. The GTPases signal through a wide variety of effectors. RhoA promotes stress fibre and focal adhesion formation, maintains adhesion to the substrate during migration, and contributes to cell contractility necessary for retracting the trailing edge (Chrzanowska-Wodnicka and Burridge, 1996). RhoA supports contractility through its effector Rho kinase (ROCK), which increases the phosphorylation of myosin II light chain (MLC) (Totsukawa *et al.*, 2000; Kimura *et al.*, 1996). Another RhoA effector participating in actin organisation is mammalian diaphanous 1 (mDia1). Rac1 is responsible for protrusive lamellipodial extension and forward movement through the control of actin polymerisation in many ways. The balance of RhoA and Rac1 signalling is alternative. In the initial phase of adhesion, RhoA activity is attenuated while Rac activity is induced. Later, RhoA activity is increased and Rac1 activity reduced. Rac1 signalling antagonises RhoA at the GTPase level, but RhoA does not influence Rac1 (Sander *et al.*, 1999). In contrast, Cdc42 regulates filopodia formation. Essential for cell polarity, Cdc42 localises lamellipodial activity to the leading edge. The common downstream effector of both Rac1 and Cdc42 is p21-activated kinase (PAK). It modulates focal adhesion disassembly and cytoskeletal dynamics through several targets. PAK mediates activation of the ERK MAPK, which stimulates the myosin light chain kinase (MLCK). Another downstream target of PAK is the c-Jun N-terminal kinase (JNK), which activates the transcription factor Jun responsible for the transcriptional regulation of many genes (Ridley, 2001). Integrin signalling regulates lipid rafts on the plasma membrane. Upon cell adhesion to the ECM, RhoA and Rac1 are targeted to the leading edge of the cells in lipid rafts. Thus, besides directly activating Rac1, integrins regulate the targeting of Rac1 and couple Rac1 to its effector PAK (del Pozo *et al.*, 2004). Similarly to Rac1, integrins regulate the localisation of RhoA to the lipid rafts at the cell front and couple RhoA

with its effector mDia1 (Palazzo *et al.*, 2004). A critical integrin signalling mediator of the process of lipid raft targeting is FAK (Palazzo *et al.*, 2004). Furthermore, targeted mutation of the β1 integrin cytoplasmic domain results in the abrogation of Rac1-mediated proliferation and survival (Hirsch *et al.*, 2002).

Contraction of the actin stress fibres that are connected to focal adhesions, endocytosis of integrins, and cleavage of the integrin–cytoskeleton linkage at the cell rear very close to integrins together induce retraction in the cell tail, allowing for the cell body to glide forward. Mechanical forces drive disassembly, loosening the connection to ECM, and microtubules by modulating contractility and mediating polarity (Webb *et al.*, 2002). Cell speed and persistence i.e. directionality of migration depend on the formation and disassembly of connections to the actin cytoskeleton, mainly the interactions of integrins and the ECM. Focal adhesion formation is mechanistically distinct from disassembly. However, both involve endocytic processes, and therefore couple to integrin recycling. It has been speculated that adhesion turnover would require a coordinated cycle of phosphorylation and dephosphorylation of the regulators, involving signalling components FAK, Src, tyrosine kinases, and phosphatases in addition to the Rho family GTPases (Webb *et al.*, 2002). In a moving cell, RhoA and the protease calpain regulate the processes of tail focal adhesion disassembly or abandonment of the substratum (Palecek *et al.*, 1997; Worthylake *et al.*, 2001). Disassembly is also regulated by the accumulation of endocytic components such as clathrin and its adaptors at focal adhesions. Microtubules facilitate clathrin localisation to focal adhesions and thus enhance disassembly (Ezratty *et al.*, 2009). In contrast to random migration, directionally migrating cells rapidly move to a certain direction. Directionality can be intrinsic or involve chemotaxis. Regulators of directional migration include microtubules, Rac1, Cdc42, integrins, and chemotactic stimuli. Culturing fibroblasts in 2D versus 3D environment has been shown to modulate Rac1 GTPase activity and change intrinsic directionality of cell migration. Specifically, changes in total Rac1 activity serve as a switch between random and directionally persistent migration. Moderate levels of active Rac1 allow cell turning, promoting the formation of several peripheral lamellae, which leads to random motility. Lower Rac1 activity relates to directionally persistent migration due to the axial lamellae and suppression of the number of peripheral lamellae and associated membrane protrusions (Pankov *et al.*, 2005).

2.1.1.3. Integrins participate in regulation of actin dynamics

In response to extracellular signals, actin-binding proteins regulate the different steps of actin cytoskeleton assembly: filament nucleation, elongation, severing, capping, and depolymerisation. Cellular actin dynamically transits between the monomeric and filamentous forms (Lee and Dominguez, 2010). Actin polymerisation, directed and dynamic organisation of a dense and branched actin network, plays a crucial role in the extension of the protruding lamellipodium as well as other cellular structures. The Arp2/3 complex and formins regulate the initiation of filament nucleation and assemble actin monomers into the elongating filament. The Arp2/3 complex regulates

the assembly of the polymerising branched, sheetlike actin network, allowing protrusive force in the lamellipodium whereas formins support elongation of linear actin and use profilin to help recruit actin monomers. The actin monomer embodies an adenosine triphosphate (ATP) binding site. During incorporation of an actin monomer to the filament, a process called nucleation, ATP is exchanged to adenosine diphosphate (ADP) that is trapped in the actin filament until depolymerisation. The filaments are polar and elongate at the plus or 'barbed' end and depolymerise at the minus or 'pointed' end. Capping of the filament seals it from further polymerisation. Thus, inhibition of capping is required to create longer filaments (Insall and Machesky, 2009; Chhabra and Higgs, 2007).

Along with the Rho GTPases, integrins regulate actin polymerisation locally. One of the major integrin signalling targets is the actin cytoskeleton, and many actin-binding proteins localise in focal adhesions. Arp2/3 is trapped to integrin adhesions through interactions with FAK and vinculin. To start actin polymerisation, the Arp2/3 complex must be activated by an actin nucleator like the Wiskott-Aldrich syndrome protein (WASP). RhoA GTPase induces the assembly of actin filaments through Diaphanous-related formins (DRFs) that directly nucleate unbranched filaments (Tominaga *et al.*, 2000). Rac1 may activate the Arp2/3 complex to nucleate actin filaments or DRFs, and regulate integrins at the edge of the protrusion, promoting recruitment and clustering of activated integrins. In addition to Rac1, also Cdc42 activates Arp2/3 to induce extension of filopodia, which act as precursors for Rac1-induced lamellipodia. Cdc42 promotes linear actin polymerisation via formins and vasodilator-stimulated phosphoprotein (VASP) involving the actin crosslinking protein fascin to organise the elongated filaments (Hynes, 2002; Legate *et al.*, 2009; Vicente-Manzanares *et al.*, 2009).

The turnover of mature focal adhesions is selective depending on the surroundings and cellular needs. Thus, mature focal adhesions are sparsely found at the cell rear. Rho family proteins act as adhesion formation conductors and the periphery of lamellipodia holds the actin dynamics regulators profilin, the Arp 2/3 complex, WASP, and VASP in close proximity of the adhesion-related components like integrins, talin, and α-actinin. The nascent cell-substratum contact sites are stabilised by these complexes (Webb *et al.*, 2002).

2.1.1.4. Integrin traffic

Integrin traffic plays an essential role in cell migration (Caswell and Norman, 2006; Pellinen and Ivaska, 2006). To migrate efficiently, cells need endocytosis to dynamically modulate their adhesion and signalling. Endocytosis not only transports integrin receptors but also shapes chemokine gradients, distributes GFRs to the leading edge, influences remodelling of FN, and assures movement cohesion in multicellular sheets by cadherin turnover (Ulrich and Heisenberg, 2009). Furthermore, integrins contribute to the trafficking of other receptors and cargos such as lipid rafts and RTKs (Caswell *et al.*, 2009). There are several mechanisms to transport integrins through the

endocytic vesicles or the endosomal recycling pathways that translocate integrins to other adhesion sites. All of the mechanisms involve several counterparts: kinases, motor proteins, cytoskeleton, and small Rab GTPases. In fibroblasts, endosomal vesicles seem to move from the cell rear directly back to the plasma membrane or to the perinuclear space and from there to the front lamellipodial base (Webb *et al.*, 2002). Subject to cell type and environment, vesicles can be internalised by clathrin-dependent, clathrin-independent, or caveolin-dependent mechanisms. Clathrin-coated pit scissors, dynamin GTPases, promote constriction and fission of vesicles. If not engaged with ECM, integrins can be endocytosed via the clathrin-independent, caveolin-dependent pathway. Caveolae are specialised forms of lipid rafts (Echarri and Del Pozo, 2006). The membrane protein caveolin is essential for Src-FAK activation in lipid rafts, triggered by β1 integrin (Wei *et al.*, 1999)

Rab GTPases regulate intracellular trafficking of integrins and other proteins. They function both in the fusion of membrane vesicles and in transporting the vesicles along cytoskeletal tracks. Integrins are endocytosed along microtubules from the cell surface and recycled back to the membrane clathrin-dependently or clathrin-independently either via the short-loop Rab4-positive or the long-loop Rab11-positive compartment important for β1 integrins. Also, targeting the endocytosed receptors for degradation is an option, but not the major purpose of integrin traffic (Ivaska and Heino, 2010). Rab5 and Rab21 bind to and activate an enzyme involved in intracellular trafficking among other cellular processes, phosphatidyl inositol 3-kinase (PI3K), and interact with integrin α subunits. Rab21 positively regulates integrin endo-exocytic traffic and movement of the vesicles along microtubules. Interfering with the function of this traffic has been shown to impair adhesion and migration of breast and prostate cancer cells (Pellinen *et al.*, 2006). Furthermore, Rab21-mediated integrin endocytosis is essential for adequate cell division, and deletion of Rab21 in cancer results in the accumulation of multinucleate cells that have undergone unsuccessful cell division (Pellinen *et al.*, 2008). Together with Rabs, the Arf family GTPases contribute to the regulation of membrane traffic among several other cellular processes. Arfs regulate vesicular traffic and organelle structure, participating in vesicle formation (D'Souza-Schorey and Chavrier, 2006). Arf6 regulates actin remodelling as well as the endocytosis and recycling of β1 integrins. Arf6-mediated internalisation of β1 integrin is a clathrin-independent process, but an adaptor of clathrin, adaptor protein 2 (AP2) assembles an indispensable complex. This AP2 complex recruited to membranes by Arf6 plays a role in post-endocytic sorting of Arf6 cargo, promoting recycling over lysosomal targeting (Lau and Chou, 2008). Inhibition of Arf6 attenuates cell adhesion and migration (Dunphy *et al.*, 2006). Activated integrin α2β1 is clustered in lipid rafts on the plasma membrane, which induces α2β1 lateral redistribution to caveolae to be internalised PKCα-dependently involving microtubules (Upla *et al.*, 2004). Actin-based motor protein myosins are implicated in many forms of motility. These include endocytosis and intracellular trafficking. Filopodia are characteristically Cdc42 GTPase-triggered long and slim cytoskeletal protrusions sensoring cell surroundings. Myosin X moves back and forth within the filopodia transporting integrins along actin filaments to filopodial tips (Zhang *et al.*, 2004). Taken together, elements of cell

cytoskeleton, namely actin filaments and microtubules, function as tracks for integrin traffic. Thus far, the role of IFs, another cytoskeletal system, in integrin endocytosis is less well studied.

Membrane trafficking has been shown to regulate directionality in cell migration. Inhibiting Rab recycling increases non-directional cell motility (Prigozhina and Waterman-Storer, 2006). The partitioning-defective (PAR) polarity complex consisting of PAR-3, PAR-6, and atypical PKC (aPKC) functions in polarised cell migration and localises at the leading edge. Integrin-binding, cargo-specific adaptor protein Numb can bind to this complex. The PAR polarity complex allows for the adaptor protein Numb phosphorylation by aPKC and subsequent release from clathrin-coated structures and integrin binding. Directional cell migration but not cell spreading requires Numb-mediated endocytosis of β integrin (Nishimura and Kaibuchi, 2007), and Numb has been shown to directly bind to the cytoplasmic domain of β integrin through its phosphotyrosine-binding (PTB) domains that can interact with the conserved NPXY (where X stands for any amino acid) motif present in most β integrin cytoplasmic domains (Calderwood *et al.*, 2003). The role of Numb in endocytosis only applies for integrin endocytosis and not for the common clathrin-dependent endocytosis process. Disabled-2 (Dab2) is another NPXY sequence-dependent clathrin adaptor protein binding to β integrin and the machinery of clathrin-dependent endocytosis. A cooperational role for Numb and Dab2 in integrin endocytosis has been proposed (Nishimura and Kaibuchi, 2007) and recently, Dab2 was shown to support clathrin-dependent β1 integrin endocytosis. Dab2 also influenced the surface levels of β1, α1, α2, and α3 but not α5 or αv integrins. The data indicate that Dab2 regulates the endocytosis of integrins disengaged from the cytoskeleton whereas Numb is localised near focal adhesions and participates in the endocytosis of integrins from disassembling adhesion sites (Teckchandani *et al.*, 2009). In contrast, PKCα directly associates with β1 integrin cytoplasmic domains and promotes β1 integrin caveolar endocytosis (Upla *et al.*, 2004; Ng *et al.*, 1999a).

Many pathogens invade cells using the endocytic machinery and integrin receptors for entry. Hence, integrins support viral uptake and infection like many other membrane receptors including the syndecan proteoglycans (Wickham *et al.*, 1993). Several viruses bear the integrin recognition site RGD motif on their surface and use it for entry in clathrin-coated pits to endosomes (Wickham *et al.*, 1993; Akula *et al.*, 2002; Acsadi *et al.*, 1994; Cseke *et al.*, 2009). Human echovirus 1 (EV1) and rotavirus are the only viruses depicted to internalise into caveolae bound to α2β1 integrin and dependent on dynamin. EV1 mediates clustering of the receptors, but does not induce an activating conformational change in the receptor as binding of the specific collagen ligand or antibody does (Jokinen *et al.*, 2010).

2.1.1.5. Contribution of integrins on protein kinase C activation

Members of the PKC superfamily are serine-threonine kinases involved in cell migration and processes of signal transduction and growth regulation. Also, their role in various integrin-dependent cellular functions is firmly established (Rigot *et al.*, 1998). Protein phosphorylation has been demonstrated to regulate the traffic of integrins in motile cells. In particular, PKC family members as well as other serine-threonine kinases have been shown to regulate integrin transport in cells, thus affecting both adhesive properties and migration (Rigot *et al.*, 1998; Roberts *et al.*, 2001; Woods *et al.*, 2004). The PKCs are divided in the best characterised classical, Ca^{2+} and lipid second messenger diacylglycerol (DAG)-dependent PKCs (α, βI, βII, γ), novel PKCs (δ, ε, η, θ) that require DAG for activation, and atypical PKCs (ζ, ι/λ) that are neither dependent on Ca^{2+} nor DAG (Disatnik *et al.*, 2002). When inactive, PKCs are auto-inhibited by a regulatory domain sequence called pseudosubstrate site. They are allosterically activated by binding of second messengers or effectors that cause phosphorylations on several possible residues and the displacement of the regulatory domain from the substrate binding pocket (Roffey *et al.*, 2009; Rosse *et al.*, 2010). Cell adhesion affects PKC phosphorylation, and in response to activation, integrins can induce phosphorylation of novel PKCs via pathways involving PI3K: via FAK (Parekh *et al.*, 2000) or via phospholipase C (PLC) (Clark and Brugge, 1995). Both novel and classical PKCs have been shown to act as positive regulators of cell adhesion and migration (Rigot *et al.*, 1998).

PKCs, above all PKCα, seem to generally promote cell migration in many cell types (Larsson, 2006). F-actin binding ezrin-radixin-moesin (ERM) proteins are implicated in cytoskeletal turnover, maintenance of cell shape, and lamellipodial protrusion. Also, they link transmembrane molecules such as adhesion receptors and ion channels to the actin cytoskeleton. Ezrin is a key structural component in Rho GTPase-regulated dynamic membrane structures lamellipodia and filopodia, functionally regulated by PKCs. PKCα associates with ezrin, leading to threonine phosphorylation and activation of ERM, and subsequent enhanced cell migration (Ng *et al.*, 2001). On cell adhesion, PKCα and PKCε localise to focal adhesions and mediate cell spreading in both integrin- and syndecan-mediated signalling pathways (Haller *et al.*, 1998). Interestingly, syndecan-4 proteoglycan binds and specifically activates only the isoform PKCα (Oh *et al.*, 1997) and translocates PKCα to focal adhesions (Lim *et al.*, 2003). In particular, PKCα and PKCε interact with β1 integrins, but integrin engagement affects only the phosphorylation state of PKCε (England *et al.*, 2001). Internalisation of β1 integrins is regulated by PKCα (Ng *et al.*, 1999a) and association of PKCα with the integrin β1 cytoplasmic tail is required for directional chemotaxis of breast cancer cells (Parsons *et al.*, 2002). Furthermore, PKCε contributes to cell migration through the control of β1 integrin traffic. PKCε controls an unidentified step in the endocytic pathway that permits integrin recycling to the plasma membrane. PKC inhibition using bis-indolylmaleimide I (BIM I) arrests PKCε together with β1 integrin in cytosolic, vesicular structures that lack endocytic markers. After removal of the

inhibitor, the vesicles disappear, and PKCε is released in presence of energy and cytosolic components (Ivaska *et al.*, 2002). In lung cancer cells, PKCε regulates the interaction of α5β1 integrin and zonula occludens 1 (ZO-1) at the leading edge of cells, subsequently triggering persistent migration (Tuomi *et al.*, 2009). PKCs regulate certain responses of hepatocyte growth factor (HGF) on its receptor Met. PKCα controls microtubule-dependent traffic of Met to the perinuclear compartment, and PKCε regulates the migratory response of HGF and Met signalling such as ERK localisation into focal complexes (Kermorgant *et al.*, 2004). Very recently, PKCα expression was implicated with poor survival in a cohort study of breast cancer patients (Lonne *et al.*, 2010) and inhibition of PKCα was demonstrated to reduce lung metastasis *in vivo* in a mouse mammary tumour model (Kim *et al.*, 2010). Activated PKCα was evidenced *in situ* in human breast tumour tissue samples, suggesting that the phosphorylation of PKC more than mere quantity of PKC may hold clinical significance in tumour progression (Ng *et al.*, 1999b). In addition, human ductal pancreatic carcinomas were shown to display high expression of PKCδ. Human ductal carcinoma PANC1 cells transfected to express PKCδ were more tumourigenic compared to control cells *in vivo* in mice. In addition, only the PKCδ expressing cells developed lung metastases. Based on results *in vitro*, the authors speculate on the possible involvement of the ERK and PI3K/Akt pathways on the PKCδ-induced pancreatic tumour growth and invasion (Mauro *et al.*, 2010). Taken together, PKCs regulate the functions and signalling of integrins. On the other hand, both integrins and syndecans may influence the activity of different PKC isoforms.

2.1.2. Cell-surface proteoglycans

Proteoglycans (PGs) are a wide family of structural proteins that interact with other cellular receptors, growth factors, cytokines, and components of the ECM. PGs take part in signal transduction of multiple cellular processes both in normal cell behaviour and in pathological conditions such as cancer and arthritis. In ECM, the large collagen fibrils are decorated by binding of PGs and glycoproteins such as FN (Heino and Kapyla, 2009; Dreyfuss *et al.*, 2009). PGs are classified in the categories of extracellular, small leucine-rich PGs, modular PGs (hyaluronan- and lectin-binding and non-hyaluronan-binding PGs), and cell-surface PGs consisting of the membrane-spanning syndecans and the glycosylphosphatidylinositol (GPI)-anchored glypicans. PGs are composed of a core protein with covalently linked glycosaminoglycan (GAG) chains. One exception to this is hyaluronan which lacks a core protein. PG protein cores are post-translationally modified. GAG chains are negatively charged linear polysaccharides of different polysaccharide types like chondroitin sulphate (CS), heparin, and heparan sulphate (HS) GAGs. Substitution of the core with combinations of one or several GAG sidechains of different subtypes provides for the specificity of the various biological functions of PGs. For example, the large cartilage PG aggrecan bears approximately 100 CS and 30 keratan sulphate GAG chains (Schaefer and Schaefer, 2010). These GAG substitutes are responsible for the enormous molecular diversity of PGs. 50-200 unit long HS chains consist of repeating disaccharides. During

the tightly controlled biosynthesis, HS chains are vastly modified and heterogeneously sulphated (Okina *et al.*, 2009). The HS chains regulate various heparin-binding growth factors. In addition to other ligands such as blood coagulation enzymes, the HS chains bind to heparin-binding sites present in ECM ligands, including FN, vitronectin, laminins, and fibrillar collagens (Beauvais and Rapraeger, 2004). Modification of the disaccharide chain interactions results in inhibition of cellular responses to ligands such as FN and some growth factors. Conversely, mere extracellular protein core domains have been shown to interact with integrin functions (Morgan *et al.*, 2007).

The syndecans are cell-surface, transmembrane PGs consisting of a core protein and covalently attached, large HS sidechains capable of engaging components of the ECM. Additionally, some of the syndecans have chondroitin or dermatan sulphate chains. Unique HS sidechain composition provides specificity through molecular interactions between the ECM and syndecan. The mammalian syndecan family comprises four members of which the focal adhesion component syndecan-4 is expressed ubiquitously and syndecan-1, -2, and -3 have restricted tissue distribution. Syndecan-1 is present early during development and on epithelial and cancer cells in adults. Syndecan-2 is found in cells of mesenchymal origin and syndecan-3 primarily in neuronal tissues (Xian *et al.*, 2010; Multhaupt *et al.*, 2009). Syndecans tend to form SDS-resistant dimers, which is probably essential for their biological roles. For a functional syndecan-4 dimer, four HS chains are required (Gopal *et al.*, 2010). Two highly conserved cytoplasmic domains C1 and C2 are a hallmark of the syndecan class. Between these two domains that are the same in each of the syndecans is a variable, central V region which is distinct and species-specific for each syndecan family member (Figure 5). The V region is speculated to be responsible for a majority of the syndecan-specific interactions and functions. The syndecan-4 dimer structure is known to rest on the ability of the V region to form a twisted clamp motif (Shin *et al.*, 2001). For intrinsic enzymatic activity, the cytoplasmic domains are too small, only around 30 amino acids long. Therefore, interactions are required for signalling (Xian *et al.*, 2010).

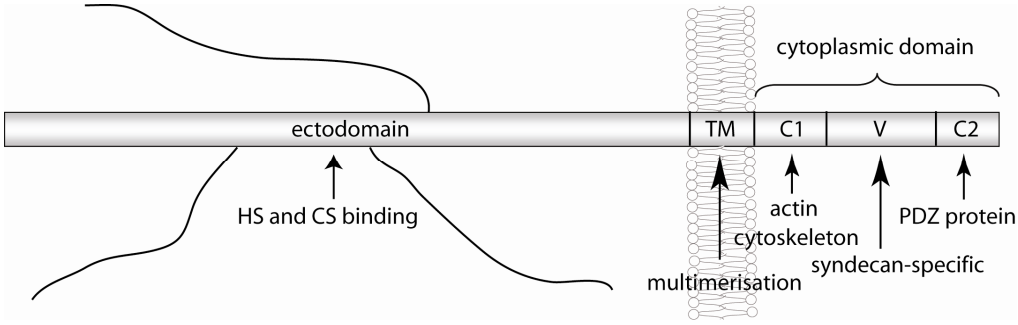

Figure 5. Syndecan structure. A schematic view presents the structure of a syndecan with three HS and/or CS GAG sidechains. Interactions of the specific regions are indicated with arrows. The structure was presented in (Couchman, 2003) in a similar manner. TM, transmembrane domain; C1 and C2, conserved regions; V, variable regions; PDZ (post-synaptic density 95; disc large tumour suppressor; ZO-1).

Single knockouts of syndecan genes in mice yield mild phenotype defects such as impaired wound healing, suggestive of redundancy among cell-surface PGs in embryonic development (Multhaupt *et al.*, 2009). Syndecans operate as integrin and cell-cell adhesion molecule co-receptors facilitating cell adhesion. In addition, they have their own direct signalling functions primarily converged to the cytoskeleton in cell-matrix interactions and matrix assembly (Beauvais and Rapraeger, 2003). They also regulate the signalling of growth factors and chemokines. In several types of carcinoma, syndecan-1 is downregulated (Beauvais *et al.*, 2004). Loss of syndecan-1 extracts epithelial cell characteristics and results in a fibroblast-like phenotype with reduced E-cadherin expression and disorganised F-actin, suggestive of a role in EMT (Kato *et al.*, 1995). On the contrary, syndecan-1 also promotes tumourigenesis. In many aggressive carcinomas, syndecan-1 expression is enhanced. Syndecan-1 has been shown to excite metastasis formation and to induce the function of Wnt-1, a secreted polarisation protein implicated in cancer and embryogenesis. Syndecan-2 is frequently overexpressed in colon carcinoma by several folds, and syndecan-4 in hepatocellular carcinomas and mesotheliomas (Beauvais and Rapraeger, 2004).

2.1.2.1. Integrin-syndecan cooperation in cell adhesion and migration

The integrin family receptors are recognised as the primary component in adhesion during developmental and pathological processes. PGs, especially syndecans, interact with ECM molecules as well, and many key functions for PGs in signalling have been established (Couchman and Woods, 1999). Syndecans bind to ECM proteins and mediate extracellular signals inside the cell, activating cytoplasmic signalling. Structural proteins, growth factors, chemokines, cytokines, and cell-surface adhesion receptors promote adhesion via binding interactions with PGs. Integrins and syndecans constitute the linkage connecting the ECM to the cytoskeleton. Binding domains for PGs and integrins lie in close proximity within ECM molecules, implicative of cooperative and modulative ligand recognition (Beauvais and Rapraeger, 2004). Syndecans and integrins collaborate extensively in cell adhesion to the ECM and together regulate focal adhesion formation, cell spreading, and directional migration (Xian *et al.*, 2010). Furthermore, both receptors are required for transduction of mechanical forces, regulation of cytoskeletal dynamics, and laying the foundation of adhesion signalling complexes in the adhesion structures: focal contacts, focal adhesions, and fibrillar adhesions. The molecular mechanisms behind the interplay between these receptor families remain obscure, but a wide repertoire of *in vitro* studies demonstrate clear synergy in signalling through protein kinases and Rho family GTPases as well as in functions *in vivo* (Morgan *et al.*, 2007). Besides the integrin and syndecan synergism in matrix engagement, both receptors can be transported in intracellular vesicles and they localise to the same endocytic vesicles. Similarly to integrins, recycling of syndecans has been shown to influence cell spreading (Zimmermann *et al.*, 2005).

Syndecan-4 connects to the cytoskeleton through several binding partners. One of them is α-actinin (Greene *et al.*, 2003) and another syndesmos, a protein that directly binds the focal adhesion adaptor protein paxillin in responce to PKC activation (Baciu *et al.*, 2000; Denhez *et al.*, 2002). The data on other syndecans than syndecan-4 present in focal adhesions are somewhat contradictory, but it seems that in concert with syndecan-4, syndecan-2 cooperates with α5β1 integrin in cell adhesion and actin cytoskeleton organisation. These receptors contribute to the formation of focal adhesions and stress fibres in an expression level-dependent manner (Munesue *et al.*, 2002; Kusano *et al.*, 2004). FN-null mouse fibroblasts have been used to identify the involvement of syndecan-4 in adhesion. Syndecan-4 collaborates with integrins in generating signals for cell spreading and for the assembly of focal adhesions and actin stress fibres when cells adhere to FN. Also, syndecan-4 as the only member of the syndecan family localises in focal adhesions together with integrins (Woods and Couchman, 1994; Saoncella *et al.*, 1999). Fibroblasts adhere and spread in response to the FN RGD site through ligation of integrins such as α5β1. In contrast, focal adhesion formation requires the heparin-binding domain of FN for syndecan-4 HS chain binding and signalling by recruitment of PKCα and subsequent downstream activation of the RhoA GTPase (Dovas *et al.*, 2006). In Chinese hamster ovary (CHO) cells, overexpression of syndecan-4 results in excessive focal adhesion formation and reduced migration (Couchman, 2003; Longley *et al.*, 1999).

Syndecan-4 and integrins are both essential for proper focal adhesion formation. Syndecan-4 engagement takes part in modulating levels of RhoA and Rac1 activity, RhoA being suppressed when Rac1 is activated (Bass *et al.*, 2007). In fibroblasts, syndecan-4 promotes persistent migration by both localisation and activation of Rac1 GTPase (Bass *et al.*, 2007) while integrins have been shown to participate only in the targeting of Rac1 to the leading edge and not on Rac1 GTP loading (del Pozo *et al.*, 2004). During initial cell spreading and upon syndecan-4 engagement (FN), Rac1 is upregulated in a PKCα-dependent manner while RhoA activity is reduced (Bass *et al.*, 2007). Integrin β1 participates in the membrane localisation of Rac1. When cells adhere to FN dependent on β1, an abrupt decrease in RhoA activity has been reported. At later stages during cell spreading, activated RhoA levels rise again (Danen *et al.*, 2005). Clustered in focal adhesions, syndecan-4 and α5β1 integrin individually contribute to the attenuation of RhoA signalling to regulate cell migration, both influencing the GTP loading of RhoA. Syndecan-4 regulates the distribution, and α5β1 integrin signalling the activity of p190RhoGAP Rho GTPase activating protein (Bass *et al.*, 2008). Thus, cooperative signalling of integrin and syndecan regulate the activity and spatial targeting of RhoA, Rac1, and p190RhoGAP (Echtermeyer *et al.*, 2001).

The relationship of syndecans and integrins has been studied in MDA-MB-231 human breast carcinoma cells, where syndecan-1 core protein participates in adhesion-mediated signalling in cooperation with αvβ3 integrin (Beauvais and Rapraeger, 2003). Syndecan-1 extracellular core directly interacts with αvβ3 and αvβ5 integrins (Beauvais *et al.*, 2004; McQuade *et al.*, 2006). PDZ (post-synaptic density 95; disc large tumour suppressor; ZO-1) proteins anchor transmembrane proteins to the actin

cytoskeleton and especially those with several PDZ domains function as scaffolds in maintaining signalling complexes (Kurakin *et al.*, 2007). Synstatin, a PDZ domain peptide inhibitor targeted against the functional part of syndecan-1 extracellular domain, impairs β integrin functions, and in a mouse model, blocks angiogenesis and mammary tumour growth (Beauvais *et al.*, 2009). During adhesion on laminin, synergistic cooperation has been observed between syndecans and α2β1 integrin (Hozumi *et al.*, 2006) as well as between α6β4 integrin and syndecan-1. The essential region in syndecan-1 for the interaction with α6β4 integrin is the conserved C2 domain, suggesting that also other syndecans may interact with α6β4 integrin (Ogawa *et al.*, 2007). A laminin-derived syndecan-binding peptide clusters and colocalises syndecan-4 and β1 integrin, leading to integrin activation and stimulated migration of keratinocytes (Araki *et al.*, 2009; Wang *et al.*, 2010). In fibroblasts and some leukocytic cells, extracellular syndecan-4 NXIP core domain triggers β1 integrin-mediated adhesion (Whiteford and Couchman, 2006) whereas syndecan-1 extracellular core domain functions as an αvβ3 integrin co-receptor to signal MDA-MB-231 cell spreading (Beauvais and Rapraeger, 2003). Raji lymphoblastoid cells lack all syndecans and grow in suspension. When transfected to express syndecan-1, these cells become adherent on syndecan-1 antibody or matrix ligands. Interestingly, in this somewhat artificial system cell binding, spreading, and signalling are transmitted by the transmembrane and extracellular core domains of the receptor, independently of the syndecan cytoplasmic tail, GAG chains, and β1 integrin (Lebakken and Rapraeger, 1996; Lebakken *et al.*, 2000). Distinct pathways for syndecans in supporting integrin-mediated adhesion have been described. One involves HS chains and cytoplasmic signalling of syndecan-4. Another pathway, mesenchymal-restricted, is independent of HS and syndecan signalling, and functions through syndecan-2 and -4 ectodomains supporting β1 integrin-mediated adhesion. The mesenchymal pathway requires RhoA GTPase and ROCK for cell spreading and focal adhesion formation (Whiteford *et al.*, 2007).

2.1.2.2. The influence of syndecans on various cell signalling functions

Significant interplay between syndecans and matrix metalloproteinases takes place in cells. Syndecan extracellular domain shedding, i.e. proteolytic cleavage by secreted and membrane-associated MMPs, terminates ECM binding and causes accumulation of the shed ectodomains to compete with intact syndecans for extracellular ligands. Shedding is thought to play a role in regulating the syndecan signal transduction from ECM to the intracellular signalling cascades. A multitude of shed syndecan-1 and -4 ectodomains are present in wounded tissue fluid, believed to regulate inflammation there and snare chemokines away from their primary receptors to offer protection for tissue against damage (Bass *et al.*, 2009). Metalloproteinases that can cleave syndecan-1 ectodomain include MT1-MMP, matrilysin MMP-7, and gelatinases MMP-2 and MMP-9 (Okina *et al.*, 2009). Likewise, studies in 3D matrices suggest a role for syndecan-1 in MMP regulation, specifically in modulation of MMP-9 activity and tumourigenesis (Gama-de-Souza *et al.*, 2008). Dependent on the syndecan-2 HS side

chains, syndecan-2 reduces MMP-2 expression and thereby suppresses metastasis, thus suggesting that metastatic potential is inversely proportional to syndecan-2 levels (Munesue *et al.*, 2007).

The ERM family proteins interact with syndecans in addition to PKCs, CD44, and other binding partners. The ERM proteins operate as crosslinkers, connecting plasma membrane proteins to the actin cytoskeleton. Ezrin binds to a conserved sequence in syndecan-2 C1 cytoplasmic domain. Ultimately, syndecan oligomerisation and phosphorylation of serine and tyrosine residues in the cytoplasmic tail influence the interaction. Regulation of the ERM binding to membrane proteins could be a mechanism for syndecans to participate in the regulation of cell shape and behaviour (Granes *et al.*, 2000; Granes *et al.*, 2003). In COS-1 monkey renal fibroblasts and Swiss 3T3 mouse embryonic fibroblasts (MEFs), endogenous syndecan-2 colocalises with cortical F-actin structures and syndecan-2 overexpression induces the formation of long filopodia, a process that requires Cdc42 GTPase activation (Granes *et al.*, 1999). FAK activation and fibroblast survival depend on interactions with the heparin-binding II domain of FN. These interactions suggest a link between syndecan-2 and the actin cytoskeleton in organising molecules within subcellular structures to regulate cell morphology and sustain cell survival (Beauvais and Rapraeger, 2004).

Syndecan-4 oligomerisation allows for the binding of PKCα. The ternary complex potentiates PKCα activity in focal adhesions. Consequently, syndecan-4 overexpression in a PKCα-dependent manner intensifies focal adhesion assembly through RhoA downstream pathway leading to Rho kinases and myosin II phosphorylation, resulting in reduced cell migration (Lim *et al.*, 2003; Dreyfuss *et al.*, 2009). Several PDZ domain-containing proteins have been shown to interplay with the syndecan PDZ domain-binding site located in the C2 region (Multhaupt *et al.*, 2009). A conformational change induced by the phosphorylation of syndecan-4 cytoplasmic serine-183 inhibits syndecan-4 oligomerisation and blocks PDZ domain binding of the scaffold protein syntenin (Koo *et al.*, 2006) which has a diversity of interaction partners it recruits to the plasma membrane (Beekman and Coffer, 2008). Originally, syntenin was identified as a syndecan cytoplasmic domain-binding protein (Grootjans *et al.*, 1997) and later confirmed to be a prerequisite for syndecan recycling from endocytic vesicles to the plasma membrane in Arf6 recycling endosomes (Zimmermann *et al.*, 2005). Syntenin recruitment during membrane protrusion formation is dependent on the dephosphorylation of syndecan-1 PDZ domain tyrosine-309, suggesting that syndecan associations with cytoskeletal components may be regulated by phosphorylation (Sulka *et al.*, 2009). Overexpression of syndecan-4 in CHO cells reduced cell migration in scratch-wound assay. Mutation of syndecan-4 serine-183 returned cell motility back to the levels of the non-syndecan-4 expressing control cells (Koo *et al.*, 2006). Therefore, phosphorylation of syndecan has been proposed as a mechanistic switch for the regulation of syndecan associations with components of the actin cytoskeleton and other functions. Importance of syndecan-4 in wound healing, scarring, and tissue repair seems evident. Healing of skin wounds is delayed in syndecan-4 knockout mice and syndecan-4 null fibroblasts migrate non-

persistently, suggestive of high Rac1 activity (Echtermeyer *et al.*, 2001) as localised Rac1 activity results in persistent migration, but overall high active Rac1 produces several protrusions and non-persistent migration. The fundamental wound healing processes are Rac1-dependent cellular migration and RhoA-associated adhesion and contractility.

2.1.3. Matrix invasion, contraction, and remodelling

The ECM characteristics vary in structure and function between tissues. In all tissues, the ECMs contain collagens, glycoproteins, and proteoglycans. Cartilage, bone, and tendon - the musculoskeletal system - has mechanical functions, while other types of the ECM support, offer elasticity, serve as intermediary for the immune surveillance, and border tissue compartments. Cell anchorage and mechanical movement are founded on the ECM. Also, the ECM plays a role in development. Cell-ECM interactions allow organised cell migration and support differentiation events, survival, and apoptosis. Furthermore, the ECM is constantly remodelled by a variety of specialised proteins and proteoglycans. Similar cellular processes are involved during invasion, wound healing, and matrix contraction. Wound healing is triggered by tissue damage and the process includes three phases. Inflammation, new tissue formation, and remodelling all aim at restoration of the wounded area. Contraction of the wound by fibroblasts is important for repair (Okina *et al.*, 2009). Cell signalling and biological responses differ during standard 2D cell culture compared to more natural 3D matrices. Depending on the matrix composition and the surrounding physical properties, such as three-dimensionality and rigidity, cells behave and anchor using different types of adhesion contacts, and show distinct morphology and molecular composition (Yamada *et al.*, 2003). As cell signalling between regular 2D cell culturing and in 3D conditions substantially differ, more of the future studies should be performed in 3D settings to mimic *in vivo* conditions. Finally, the *in vivo* physiological relevance should be carefully evaluated when drawing conclusions from results obtained in the traditional 2D cell culture systems (Yamada *et al.*, 2003).

Much of the integrin signalling studies have been conducted on rigid material such as the tissue culture plastic, which is not representative of an *in vivo* environment. In 3D cell culture conditions, stiffness among other special characteristics influences cell adhesion, migration, proliferation, and survival (Legate *et al.*, 2009). When moving through 3D matrix, cells are surrounded by the ECM and the attachments in 3D matrix differ from the 2D focal and fibrillar adhesions in their molecular composition and in the enhanced association with physiological responses of cells, namely adhesion, migration, proliferation, and acquisition of spindle-shaped morphology. On 3D matrices, cells adhere, migrate, and proliferate faster and show low levels of FAK major tyrosine-397 phosphorylation (Cukierman *et al.*, 2001) and increased amount of activated MAPK (Damianova *et al.*, 2008). Many of the proteins that the adhesion sites in 2D and 3D contain are the same, but the 3D matrix adhesions lack tyrosine-phosphorylated FAK and $\alpha v\beta 3$ integrin. In addition, the 3D matrix adhesions are long

and slender in shape and settled in parallel to the extracellular fibres unlike the compact focal adhesions and the fibrillar adhesions with punctuate appearance in 2D conditions (Cukierman *et al.*, 2001).

3D matrices such as collagen gels provide a system closer to the natural conditions compared to the flat 2D cell culture substrates. However, the physiological 3D environments, tissues *in vivo*, comprise various molecules and show far more organised fibrillar architecture. Multiple artificial systems exist for studying cell behaviour in conditions mimicking connective tissues *in vivo*. To acquire matrices with such physiological properties, for example cryosections of frozen embryos can be utilised. Another means of production is to allow certain fibroblasts to remodel the matrix for several days. In both systems, cellular contents are extracted from the matrix before the actual study (Yamada *et al.*, 2003). Isometric tension in unreleased 3D matrices should be taken into consideration because it affects cellular signalling as dramatically as for example deciding between fibroblast proliferation and quiescence (Fringer and Grinnell, 2001). Tension is indispensable for FN deposition (Halliday and Tomasek, 1995) and actin polymerisation to stress fibres. Finally, tension stretches, modifies, and regulates the exposure of the ECM proteins in favourable conformation of specific integrin binding (Cukierman *et al.*, 2002).

2.1.3.1. Invasion and metastasis

Metastatic and invasive spread of tumour cells to distant organs is the primary reason for cancer threat and mortality. Drastic phenotypical and biochemical changes occur when a normal cell acquires invasive properties of a cancer cell (Hanahan and Weinberg, 2000). To begin the seeding process of metastasis, cancer cells need to activate EMT and many signalling pathways. During complete metastasis, several steps are taken. First, the tumour cells detach from the primary tumour mass and invade into the surrounding tissue. For dissemination throughout the body, cells intravasate into blood or lymphatic vessels, fighting for life to survive the immune surveillance and the hardships of circulation. At the secondary site, cancer cells extravasate into the specific target organ. Special efforts are required for growing in a foreign tissue environment that provides a different collection of growth and survival factors (Yilmaz *et al.*, 2007; Yilmaz and Christofori, 2009).

Studies on the discovery of functional analysis-coupled gene expression profiles, so called signatures, that are associated with metastasis, have inspired a diversity of clinical screening programmes (Segal *et al.*, 2005; Buckhaults, 2006; Christofori, 2007). The findings of a microarray screen in breast cancer cell line MDA-MB-231 that exhibits lung-specific metastasis elucidate a prominent role for four genes of the lung metastasis signature in primary tumour growth, intravasation, lung colonisation, extravasation, and metastasis outgrowth. The products of these four genes are the epidermal growth factor receptor (EGFR) ligand epiregulin, MMP-1, MMP-2, and the prostaglandin-synthesising enzyme cyclo-oxygenase-2 (COX-2) that is known to mediate wound healing and inflammatory responses. Concurrent genetic or

pharmacological manipulation of the function of these four genes collectively impedes vascular remodelling functions and thus abrogates the metastatic potential of these cells (Gupta *et al.*, 2007). In a similar microarray study, mediators of blood-brain barrier passage were identified in clinical cancer samples from patients with brain metastases: EGFR ligand HBEGF, COX-2, and one factor specific for brain metastasis only, α2,6-sialyltransferase, whose expression is normally restricted to brain (Bos *et al.*, 2009). Most highly overexpressed genes in bone metastatic populations included genes encoding interleukin-11 (*IL11*), connective tissue-derived growth factor (*CTGF)*, bone-homing chemokine receptor (*CXCR4)*, and MMP-1 (Kang *et al.*, 2003). Exploitation of microarray screening studies is increasing. The published microarray data are collected into data banks, which can be utilised for *in silico* meta-analyses of gene expression (Kilpinen *et al.*, 2008). Many intrigueing questions remain unanswered, but the expanding interest in microarray screening undoubtedly will shed light on the secrets of metastasis, and narrow the deficiency of efficient treatments to prevent cancer metastasis in the near future.

2.1.3.2. Cell contractility

Signalling pathways activated by cells in 3D environments differ from those in 2D culture. Collagen gel assays are used to study cell behaviour and matrix remodelling in 3D *in vivo*-mimicking conditions, representing situations such as wound healing. The ability of cells to contract the matrix reflects the level of remodelling. In addition to wound healing, cell contraction is needed for migration and thus, invasion and metastasis. Integrins play a major role in modulating the pathways that regulate ECM remodelling and actomyosin contraction force. For cells migrating on 2D substrate, RhoA and ROCK activity regulation is crucial to exert traction forces. Subsequent MLC phosphorylation plays a key role in increasing cell contraction (Schoenwaelder and Burridge, 1999; Larsen *et al.*, 2006). Integrin-dependent 3D collagen gel contraction is facilitated by serum components through different signalling pathways. In 3D collagen culture, α2β1 integrin mediates transport of collagen fibres and thus induces matrix contraction dependent on non-muscle myosin II-B (Meshel *et al.*, 2005). Platelet-derived growth factor (PDGF) stimulates contraction through PI3K and myosin II. Lysophosphatidic acid (LPA) induces contraction without myosin II, in contrast mediated by monomeric G protein $G\alpha_1$ (Abe *et al.*, 2003). However, contractility of floating collagen matrix is always Rho effector dependent. The PDGF-led pathway depends on ROCK whereas the pathway induced by LPA is ROCK-independent (Lee *et al.*, 2003). Instead, LPA-involved signalling that stimulates contraction requires another Rho effector, mDia1. Ultimately, the signalling pathways of PDGF and LPA that both induce floating matrix contraction converge on PAK1 and its downstream effector, actin depolymerising protein cofilin-1 (Rhee and Grinnell, 2006). Modulation of matrix contraction involves interactions between several cellular signalling mechanisms. In human foreskin fibroblasts, downregulation of the cytoskeletal protein tensin2 inhibited 3D collagen contraction and reduced Rho activity. There were no effects on FN matrix assembly even though tensins are the major cytoskeletal component of fibrillar adhesions. The mechanism for tensin2 is

regulation of the tumour suppressor gene deleted in liver cancer 1 (DLC1) RhoGAP activity in focal adhesions (Clark *et al.*, 2010). Syndecans also contribute to matrix contraction in several ways. They function in cooperation with integrins through interactions with actin. As discussed earlier, syndecan-4 associates with α-actinin (Greene *et al.*, 2003) and syndesmos, a syndecan-4 cytoplasmic domain interactor (Baciu *et al.*, 2000). Also, syndecan-4 plays a role in the incorporation of the muscle-specific isoform of actin, α-smooth muscle actin (α-SMA) into stress fibres (Chen *et al.*, 2005) and signals through PKC (Oh *et al.*, 1998).

2.1.3.3. Matrix remodelling by matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a big, 24-member family of zinc-dependent protein-cleaving endopeptidases. They are either secreted or anchored on the cell membrane as proenzymes, pro-MMPs, and activated by the removal of an amino-terminal propeptide. Stringently on multiple levels controlled ECM remodelling by MMPs is essential in many physiological events and developmental processes such as morphogenesis, tissue remodelling, angiogenesis, and wound healing as well as in maintenance of tissue homeostasis. Likewise, MMPs are strongly associated with tumourigenesis. Collectively, MMPs are capable of degrading most components of the basement membrane and the ECM. MMPs thus degrade barriers surrounding the tumour as well as open up tunnels for tumour cell invasion and spread. Besides the turnover of physical hindrance, proteolytic ECM degradation by MMPs modulates the tumour microenvironment, regulates signalling pathways, and contributes to tumour cell migration and invasion in various other ways. Examples of such roles are release of growth factors captured by the ECM, promotion of angiogenesis, remodelling of the ECM components into forms favoured by motile cells, disruption of epithelial cell-cell interactions, and proteolytic interference with bioactive molecules by exposure, cleavage, or masking of functional epitopes (Friedl and Brocker, 2000; Vu and Werb, 2000; Yamada, 2003; Kessenbrock *et al.*, 2010).

According to their substrate specificity, sequence homology, and function, MMPs can be divided into distinct subgroups of which only few are discussed here. The collagenases are active against fibrillar collagen and include MMP-1, MMP-8, and MMP-13. The gelatinases, MMP-2 and MMP-7, are important in development but also for cancer progression. They preferentially degrade denatured collagens (gelatin) and basement membrane components. The smallest MMPs, the matrilysin family consists of MMP-7 and MMP-26 (Vu and Werb, 2000; Yamada, 2003; Kessenbrock *et al.*, 2010). Membrane-type MT1-MMP (alternatively called MMP-14) associates with the cell membrane via a C-terminal transmembrane domain (Urena *et al.*, 1999). In 3D conditions, tumour cell proliferation (Hotary *et al.*, 2003), cell shape change from adhesive to migratory phenotype, and migration through the ECM (Sabeh *et al.*, 2004) have been shown to depend on MT1-MMP-driven matrix remodelling. Explicitly, MT-MMPs are responsible for protease-dependent transmigration due to their ability to remodel the basement membrane which is a specialised form of ECM (Hotary *et al.*, 2006).

Interactions of MMPs with their naturally occurring inhibitors, the family of tissue inhibitors of metalloproteinases (TIMPs), at sites of integrin and MMP colocalisation have been speculated to play a regulatory role in MMP activation (Stefanidakis and Koivunen, 2006). The net balance between MMPs and TIMPs probably plays a major role in determining the proteolytic potential of tumours. In addition to TIMP inhibition, MMPs are blocked by diverse endogenous inhibitors (Folgueras *et al.*, 2004), and the expression of MMPs is influenced by alterations in the architecture of the actin cytoskeleton (Terranova *et al.*, 1984; Werb *et al.*, 1986; Seftor *et al.*, 1992). The subcellular mechanisms to regulate pericellular MMP activity and localisation remain obscure. In keratinocytes, the mechanism is MMP-9 mRNA stabilisation by α3β1 integrin (Iyer *et al.*, 2005). Also, the MT1-MMP autolysis product blocks pericellular proteolysis and thus suggests a feedback mechanism (Tam *et al.*, 2002). MMP overexpression has been emphasised to correlate with advanced cancer development and metastasis (Hofmann *et al.*, 2005; Deryugina and Quigley, 2006). However, MMPs have been suggested to play also protective roles throughout cancer progression, altering between tumour enhancer and repressor (Andarawewa *et al.*, 2003), or even guarding the host from metastasis (Balbin *et al.*, 2003; Hamano *et al.*, 2003; Gutierrez-Fernandez *et al.*, 2008).

Collagen fibrils and other components constitute a dense, crosslinked ECM that requires proteolytic activity from cells to invade (Hanahan and Weinberg, 2000). Integrins function as receptors for many proteases, including MMPs. Integrins and MMPs interact at the leading edge of migrating cells and in membrane protrusions (Munshi and Stack, 2006; Wolf and Friedl, 2009). Especially, they cluster and restrict proteolysis in the membrane invaginations, caveolae (Puyraimond *et al.*, 2001; Galvez *et al.*, 2004), and in specialised ECM-degrading membrane protrusions, invadopodia, in invasive cells (Poincloux *et al.*, 2009; Yamaguchi *et al.*, 2009). MT1-MMP is a key enzyme for pericellular proteolysis during individual cell invasion into 3D matrix and subsequent collective migration (Wolf *et al.*, 2007). MT1-MMP has been shown to localise in the invasive breast tumour front (Ueno *et al.*, 1997) and colocalise with β1 and αvβ3 integrins in endothelial cells (Galvez *et al.*, 2002). Collagen type I binding to its cell surface receptors can alter MT1-MMP function by blockade of dynamin-dependent internalisation of MT1-MMP via clathrin-coated pits (Lafleur *et al.*, 2006). MT1-MMP can proteolytically activate αvβ3 integrin. Furthermore, αvβ3 integrin is capable of modulating MMP-2 activity by direct binding (Stefanidakis and Koivunen, 2006). α2β1 integrin has been shown to upregulate MMP-1 expression in response to collagen (Riikonen *et al.*, 1995). Implicitly, MMP-1 binds α2β1 integrin via the integrin αI domain (Stricker *et al.*, 2001). Also, α2β1 upregulates p38α MAPK in 3D collagen and subsequently induces MMP-13 epression (Ravanti *et al.*, 1999). 3D collagen culture as well as bead-immobilised α2β1 and α3β1 integrin antibodies induced MT1-MMP expression in ovarian carcinoma cells (Ellerbroek *et al.*, 2001; Vogel, 2001). In invasive breast cancer cells, 3D collagen culture induced MT1-MMP expression and proMMP-2 activation (Gilles *et al.*, 1997). α2β1 integrin is upregulated in metastatic melanoma. It has various roles in melanoma progression, such as adhesion on collagen type IV, migration on collagen type I, and contraction and

remodelling of collagen type I (Klein *et al.*, 1991; Knutson *et al.*, 1996; Schon *et al.*, 1996). In human melanoma cells, the clustering of α2β1 integrin utilising triple-helical peptide ligands induced both the proteolytic potential and the expression of MMP-1, -2, -3, -13, and MT1-MMP, suggesting a highly invasive phenotype (Baronas-Lowell *et al.*, 2004). In summary, MMPs are essential in cancer progression and they are linked with integrin signalling.

2.2. EPITHELIAL-MESENCHYMAL TRANSITION (EMT)

During embryogenesis and organ development, cells within certain epithelia change between epithelial and mesenchymal states via the processes of epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET). Mesenchymal cells contribute to tissue repair and pathological processes, namely tissue fibrosis, tumour invasiveness, and metastasis. In contrast to polarised epithelial cells that normally attach to the basement membrane, the characteristics of mesenchymal cells include enhanced migratory capacity, invasiveness, resistance to apoptosis, and increased production of the ECM components. MET has not been studied as intensively as EMT. The best detailed MET process known is kidney morphogenesis. Several molecular processes are related in the initiation and the successful completion of an EMT. These include activation of transcription factors, expression of specific cell surface proteins, expression and reorganisation of cytoskeletal components, production of enzymes destined to degrade ECM, and changes in the expression of specific microRNAs. EMTs have been subtyped into three categories based on the distinct biological settings in which they occur. Type 1 represents EMT during embryogenesis, implantation, and organ development. Tissue regeneration and organ fibrosis are associated with type 2 EMT. Lastly, type 3 EMT occurs in cancer progression and metastasis (Figure 6). EMT-like processes are additionally implicated in response to physiological injury. For example keratinocytes acquire a phenotype known as the "metastable" state during wound healing. Another example is the postovulatory healing of ovarian surface epithelium in each menstruation cycle. The functional consequences that the EMT subtype phenotypes carry differ notably, but the genetic and biochemical elements underlying and thus enabling EMT are common. Activation of EMT processes has been implicated in the acquisition of malignant phenotypes by epithelial cancer cells *in vitro*, but due to lack of evidence in clinical samples, the significance of EMT in human cancer tissues was doubted until recently (Kalluri and Weinberg, 2009; Thiery *et al.*, 2009).

EMT plays a major role both during developmental and pathological conditions. Approximately 90% of human cancers have origins in epithelial tissue. Carcinoma (epithelial cancer) becomes deadly through local invasion and development of metastasis, the clinically most relevant processes. EMTs have been described in stationary, highly differentiated epithelial cells that detach and become motile. Similar changes apply for invasion as well (Christofori, 2006). The EMT process allows for epithelial cells to loosen their E-cadherin-dependent cell-cell junctions and survive in an anchorage-independent environment, penetrate the basal lamina barrier into the

surrounding tissues, and acquire effective motility to spread in the adjacent tissue and disseminate over long distances to develop distant metastasis. E-cadherin is believed to be a master regulator of EMT as the modulation of E-cadherin expression alone can initiate and complete either EMT or MET. EMT is a reversible process and during secondary tumour formation, the disseminated cells get rid of mesenchymal properties and acquire an epithelial phenotype via MET. EMT-derived cancer cells in secondary colonies need to adapt to the new microenvironment. Typically they still resemble their origins, the primary tumour cells (Guarino, 2007; Acloque *et al.*, 2008). Tumour budding is a phenomenon biologically closely related to EMT and distinct from invasion. During tumour budding, single cells or small aggregates comprising of less than five cells detach from a tumour and migrate into the adjacent stroma. Investigations addressing tumour budding at the invasive front of colorectal adenocarcinomas eventually provided morphological evidence of the status of EMT in cancer progression (Prall, 2007). The invasive fronts of colorectal carcinomas as well as other human tumours show characteristics of EMT (Brabletz *et al.*, 2001) and synchronous occurrence of both EMT-dependent and EMT-independent invasion has been suggested (Wicki *et al.*, 2006; Wyckoff *et al.*, 2007).

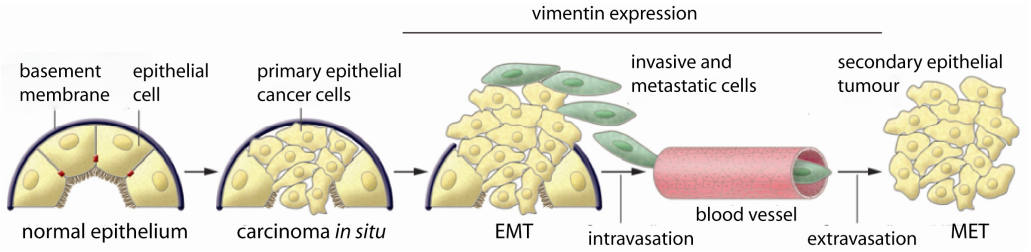

Figure 6. EMT in cancer progression. Several stages contribute to the progression from normal epithelium to invasive carcinoma. The malignant phase of tumour growth and progression to metastatic cancer involves EMTs (denoted as light yellow to darker green cells), while the formation of metastases may involve METs (green to yellow cells) for reversion to the epithelial phenotype. Adapted from (Kalluri and Weinberg, 2009).

2.2.1. Initiation of EMT

A repertoire of changes is essential for EMT. Cells lose epithelial-specific junctional components such as E-cadherin and keratins and acquire re-expression of the mesenchymal markers vimentin and N-cadherin. As a result of the EMT characteristics, cells erase cell-cell contacts, weaken the cell-ECM adhesions, and become highly motile (Frixen *et al.*, 1991). Other proteins typically expressed in mesenchymal cells include α-SMA, desmin, PDGF receptor, and αvβ6 integrin. Also, mesenchymal cells produce certain proteases (MMP-2, MMP-9) and FN. Several signals produced by tumour cells or the surrounding microenvironment induce EMT (Yang and Weinberg, 2008). Findings imply that growth factors and their receptors,

cytokines, microRNAs, and MMPs as well as membrane receptors, intracellular molecules, external agents, and even changes in the ECM composition (Shintani *et al.*, 2008) all participate in the regulation of EMT induction. Loss or downregulation of E-cadherin is frequently observed in carcinomas, but not all EMT-like cells are devoid of E-cadherin expression. Other mechanisms that interfere with its function, such as phosphorylation or mutation, are sufficient for EMT (Birchmeier *et al.*, 1996). An E3 ubiquitine ligase called Hakai binds to the tyrosine-phosphorylated form of E-cadherin and induces its endocytosis and degradation (Fujita *et al.*, 2002). Cytoplasmic β-catenin links cadherins to the actin cytoskeleton. During EMT, β-catenin translocates from the cytoplasm to the nucleus and regulates gene expression by directly interacting with transcription factors such as the lymphoid enhancer-binding factor 1 (LEF-1) (Behrens *et al.*, 1996). Alongside with the eradication of cell-cell adhesion, another crucial step for EMT is the loss of cell polarity. Three different protein complexes PAR, Crumbs, and Scribble correspond to the apical-basal polarity. Components of these complexes are regulated by EMT-inducers, in particular the zinc-finger E-box binding homeobox (Zeb) and the Snail factors (Moreno-Bueno *et al.*, 2008). Other mechanisms to initiate EMT include reorganisation of the actin and the IF cytoskeleton, activation of signalling pathways in response to ligand engagement, and regulation of the proteolytic machinery involved in matrix degradation, especially MMPs (Heino, 1996). MMP-3 for example triggers EMT through reactive oxygen species, which induces the transcription factor Snail expression (Lochter *et al.*, 1997; Radisky *et al.*, 2005). In addition, MMP-13 triggers EMT when induced by the fibroblast growth factor 1 (FGF1) (Billottet *et al.*, 2008).

The major oncogenic pathway signatures found in cancer, such as Myc, Ras, Src, and β-catenin pathways, are activated in a high percentage of human neoplasms (Bild *et al.*, 2006) and the same pathways are implicated in EMT induction. Genetic changes such as mutations produce oncogenes with gain of function and tumour suppressor genes with loss of function, each change conferring growth advantage and acquirement of tumourigenic competence (Hanahan and Weinberg, 2000). In the EMT model systems such as cancer cell lines, Src rarely is mutated, but rather oncogenically activated. Overexpressed or hyperactivated Src may induce cell growth and survival as well as promote reorganisation of the actin cytoskeleton and loss of cell-cell and cell-matrix adhesion (Yeatman, 2004) due to the activation of Ras/MAPK, PI3K/Akt, and signal transducer and activator of transcription 3 (STAT3) cascades. STAT3 in turn participates in the Myc mitogenic pathway (Guarino, 2010) and enhances the production of vascular endothelial growth factor (VEGF) and the consequent angiogenesis (Ling and Arlinghaus, 2005). Src is known to influence the expression of proteases such as MMPs via several pathways involving PKC, ERK, PI3K, Rac, and paxillin. Engagement of α2β1 integrin has been shown to activate MMPs via promoting activation of the FAK/Src/paxillin/Rac/JNK pathway (Van Slambrouck *et al.*, 2009), ERK, and p38 MAPKs (Ravanti *et al.*, 1999). Src may inhibit the endocytosis of MT1-MMP and thus increase its cell-surface amount, resulting in the activation of MMP-2 (Wu *et al.*, 2005). Interaction of collagen type I with β1 integrin recruits FAK and Src. The FAK-Src complex phosphorylates β-catenin and suppresses

RhoA acitivity through p190RhoGAP phosphorylation, leading to enhanced focal adhesion turnover in migrating cells. Subsequently, E-cadherin complexes dissolve, cells dissociate from each other, and an EMT/invasive phenotype is promoted (Koenig *et al.*, 2006). Indeed, Src activation is a potent EMT inducer. Elevated Src correlates with phosporylation of the E-cadherin-β-catenin complex (Behrens *et al.*, 1993). Phosphorylation of the complex by Src dissociates β-catenin and unmasks the region targeting E-cadherin for degradation. β-catenin is released into the cytoplasm for translocation to the nucleus. Thus, Src enhances β-catenin transcriptional activity (Lilien and Balsamo, 2005). β-catenin regulates the expression of several EMT-involved genes including Myc, members of the Snail family, vimentin, and matrix-degrading proteases (Guarino *et al.*, 2007).

Integrin receptors play an important role not only in adhesion and migration, but also in invasion, which requires altered cellular interactions with ECM. Integrins act in a similar way to the tumour suppressor genes downregulated during transformation. Spatially regulated integrin expression could lead to the formation of metastasis. Numb governs directional cell migration by controlling integrin traffic. Furthermore, cell polarity and cell-cell adhesion are regulated by Numb. It engages the PAR3-PAR6-aPKC polarity complex and through direct interactions with its PTB domain, Numb localises E-cadherin to cell-cell junctions. Src-mediated phosphorylation disentangles Numb from these molecular partners, resulting in the perturbation of cell polarity and the disassembly of E-cadherin adhesion complexes. Consequences are early EMT events: weakening of cell-cell adhesion, loss of polarity, and enhanced migration (Wang *et al.*, 2009).

Members of the transforming growth factor-β (TGF-β) family of cytokines are the main and best-studied EMT-inducers. TGF-β promotes EMT through many different signalling pathways. These mechanisms include direct phosphorylation of TGF-β transcriptional effectors, the Smads and certain cytoplasmic proteins regulating cell polarity and tight junction formation, for example the cell polarity protein PAR6A. Furthermore, TGF-β may act on several signalling pathways to induce EMT, such as Ras, RTK, or integrin signalling (Polyak and Weinberg, 2009). However, why TGF-β-induced EMT encourages carcinoma cells to get off and invade is not well understood. β5 integrin has been shown to contribute to TGF-β-induced EMT and tumourigenic potential of carcinoma cells. Also the αv subunit was shown to be essential but β1 integrin was dispensable (Bianchi *et al.*, 2010). One of the mechanisms of TGF-β to evoke invasive phenotype is to amplify the expression of a series of proteases including MMP-1, MMP-3, MMP-9, MMP-10, and MT1-MMP (Uttamsingh *et al.*, 2008). TGF-β functions as a tumour suppressor in normal cells, where it maintains homeostasis and regulates cell proliferation and matrix deposition. In these cells, TGF-β-induced EMT results in emphasised actin stress fibres and strong focal adhesions, subsequently preventing cell migration and invasion (Bianchi *et al.*, 2010). Controversially, high levels of TGF-β and activated Ras signalling are found in advanced cancers (Malumbres and Barbacid, 2003) and these pathways have been shown to interact in EMT: hyperactivated Ras complements the TGF-β-induced EMT

effects by the enhancement of cell invasion (Figure 7) (Safina *et al.*, 2009). *In vivo*, transiently and locally activated TGF-β signalling switches cells from collective to amoeboid, single cell motility. Blockade of TGF-β signalling impairs single cell movement, but allows collective cell migration. Collectively invading cells are capable of penetrating lymphatic vessels, but not the vasculature. Therefore, TGF-β signalling contributes to the selected mode of migration and thereby affects whether cancers metastasise via lymphatic or blood vessels (Giampieri *et al.*, 2009).

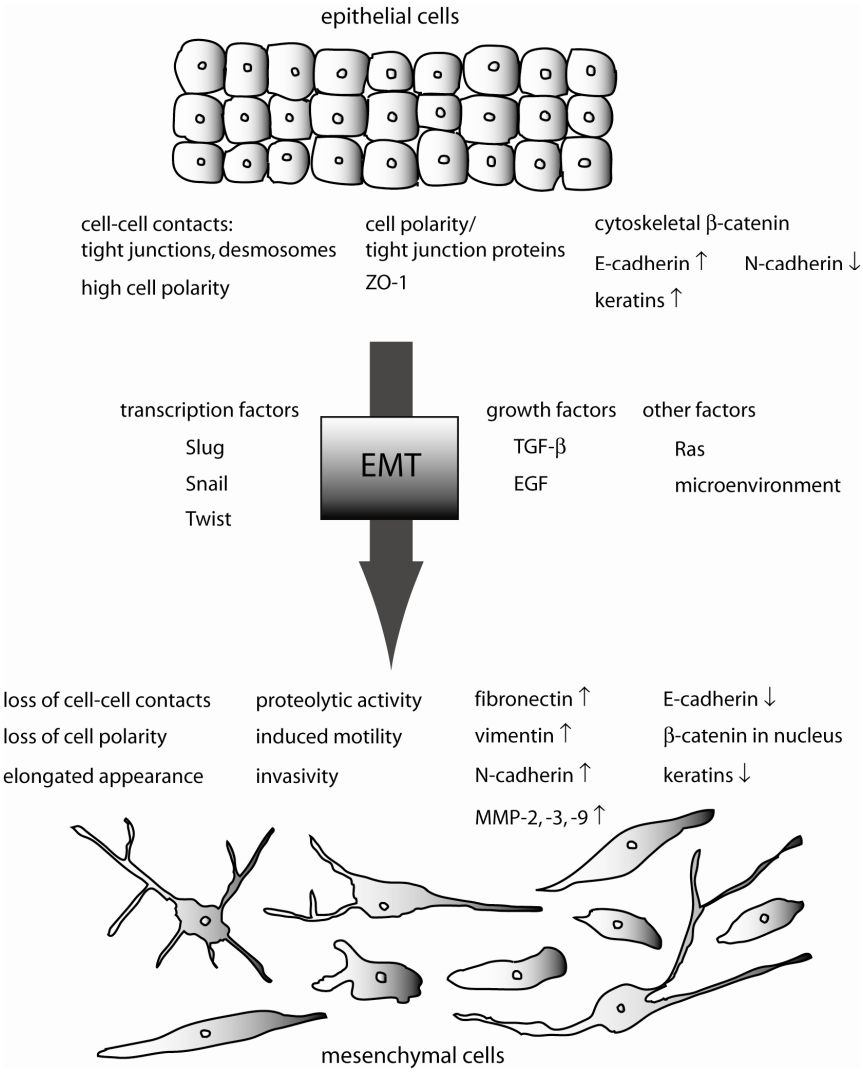

Figure 7. Summary of the major players and the characteristic properties of EMT. Several factors induce the process of EMT, and characteristics of EMT include a wide array of changes in gene expression and cellular functions.

2.2.2. Transcription factors and other EMT-inducers

Transcription factors induce EMT triggered by growth factors. As one of the mechanisms for growth factors to influence EMT, they induce or activate EMT-inducing transcription factors that repress E-cadherin indirectly of directly by binding to the promoter. These transcription factors include Snail (*SNAI1*), Slug (*SNAI2*) (Thiery, 2002), Zeb1, Zeb2/SIP1 (Chua *et al.*, 2007), E47 (Moreno-Bueno *et al.*, 2006), Krüppel-like factor 8 (KLF8) (Wang *et al.*, 2007), Twist (Yang *et al.*, 2004), goosecoid, E2.2 (Sobrado *et al.*, 2009), and FOXC2 (Mani *et al.*, 2007). The cooperation between different transcription factors has been suggested as a hallmark of EMT induction (Kang and Massague, 2004; Peinado *et al.*, 2007; Weinberg, 2008). Many of these correlate with disease relapse and survival in patients with breast, colorectal, and ovarian cancer, indicating that EMT leads to poor clinical outcome. Also, EMT profiles are linked with certain clinicopathological parameters such as the histological grade and the tumour subtype (Thiery *et al.*, 2009).

Activated H-Ras has been shown to induce a mesenchymal phenotype in MCF10A cells by others before us. Oncogene-induced EMT in these cells leads to mesenchymal morphology and importantly, RhoA activation. Subsequently, MLC phosphorylation levels elevate and contractility increases (Zhong *et al.*, 1997). MLCK is also a direct substrate of the ERK MAPK during cell migration, and ERK is constitutively active in Src-transformed cells. Src kinase induces EMT in cooperation with integrin-dependent signalling to suppress E-cadherin function and to induce the mesenchymal-like state. ERK and MLCK are critical downstream mediators of Src on the assembly of peripheral adhesion sites and suppression of E-cadherin. Src guides phosphorylated ERK and MLC to accumulate in the new integrin-dependent adhesion sites, and induces vimentin expression and E-cadherin dissociation from cell-cell contacts (Avizienyte *et al.*, 2004)

2.2.3. The intermediate filament protein vimentin

The cytoskeleton comprises three major components: actin filaments, intermediate filaments (IFs), and microtubules. Depending on cell type and tissue, cells express various IF networks comprising the nuclear IF lamins and cytoplasmic IFs including keratins, vimentin, neurofilaments, peripherin, nestin, and desmin, regulated by multiple associated proteins. All IFs constitute of a central rod domain flanked by an amino-terminal head and a carboxyl-terminal tail domain. IFs provide scaffolding and cytoprotective integrity against mechanical and non-mechanical stresses. Functions extend to organelle regulation, intracellular trafficking, and several tissue-specific roles. IFs are also linked to different integrin-mediated adhesion sites. Hemidesmosomes interconnect keratin IFs and ECM through integrins and attach cells to the basement membrane; vimentin-associated matrix adhesions (VAMs) associate vimentin and actin to integrins, and vimentin may connect with integrins outside of focal adhesions in lipid rafts at cell borders. Several human diseases including skin diseases, muscular dystrophies, and amyotrophic lateral sclerosis are linked with IF

mutations. Dynamic interactions with IF-associated proteins and post-translational modifications (phosphorylation, transglutamination, glycosylation, and caspase-mediated proteolysis) regulate the properties and interactions of IFs. Under normal physiological conditions, ubiquitination and proteasomal degradation are mechanisms of IF turnover (Ivaska *et al.*, 2007; Toivola *et al.*, 2005; Toivola *et al.*, 2010).

Vimentin is the main IF protein of the mesenchymal cell cytoskeleton, present in a wide selection of cell types: fibroblasts, endothelial cells, leukocytes, and macrophages (Eriksson *et al.*, 2004). In non-pathological conditions, vimentin is expressed during developmental processes. In addition, mesenchymal cells in adults express vimentin in the central nervous system (Eddleston and Mucke, 1993) and in all connective tissue as well as upon injuries (neural, renal) (Perlson *et al.*, 2005; Vansthertem *et al.*, 2010) and in myofibroblasts in the regenerating muscle (Vaittinen *et al.*, 2001). Vimentin participates in multiple cellular processes such as cell adhesion, migration, cell cycle progression, apoptosis, and lipid transport. Tissue graft rejection involves both alloimmunity and autoimmunity (conditions where the body gains immunity against foreign cells or its own cells, respectively). Patients with cardiac (Jurcevic *et al.*, 2001; Barber *et al.*, 2004) or renal (Carter *et al.*, 2005; Jonker *et al.*, 2005) transplantion develop autoimmunity for vimentin. Citrullination is a post-translational modification, which changes the amino acid arginine to citrullin, subsequently affecting protein folding. Antibodies for citrullinated vimentin are detected almost exclusively in patients with rheumatoid arthritis. The antibodies are present even before the onset of the disease. Thus, following the levels of these autoantibodies is useful diagnostically and when monitoring the effect of therapy on the disease pathogenesis (El-Gabalawy and Wilkins, 2004; Pruijn *et al.*, 2010). Furthermore, vimentin is upregulated in EMT and linked with a highly motile and invasive phenotype. Thus, vimentin expression is frequently used as a mesenchymal marker of transformation. In aggressive breast cancer cell lines, vimentin correlates with EMT-associated changes such as loss or reduced expression of E-cadherin and keratins and upregulation of other mesenchymal markers N-cadherin, FN, tenascin C, and PGs (Kokkinos *et al.*, 2007). Additionally, when categorising breast cancer cell lines according to microarray profiling studies, vimentin expression was identified as a marker of Basal B-like subtype. Gene expression patterns of the cell lines were shown to reflect most of the important genomic abnormalities and resulting transcriptional consequenses that are found in primary breast tumours. The Basal B-like cell lines were shown to possess high invasive activity (Neve *et al.*, 2006), and the Basal-like subtype highly correlates with poor clinical outcome (Sorlie *et al.*, 2001).

For long, the vimentin knockout mice seemed to have no obvious phenotype defect and instead developed and reproduced normally (Colucci-Guyon *et al.*, 1994). Later on, many studies analysing the vimentin knockout mice in detail have emphasised the specific functions of vimentin. Vimentin influences for example glial cell development (Colucci-Guyon *et al.*, 1999), adult neurogenesis (Larsson *et al.*, 2004), migration and contractility (Eckes *et al.*, 1998), both adult and embryonic wound healing (Eckes *et al.*, 2000), mechanotransduction of shear stress (Henrion *et al.*, 1997), modulation of

vasodilation (Terzi *et al.*, 1997), and leukocyte traffic (Nieminen *et al.*, 2006). Studies in mice demonstrated a role for vimentin in the eye lens. A point mutation or deletion in the coiled domain of vimentin was planned analogous to the keratin mutations that are known to cause many IF disorders such as epidermolysis bullosa and desminopathies. Expression of these vimentin mutations disrupted the endogenous vimentin filaments and induced aggregation formation in fibroblasts. In mice, vimentin aggregates accumulated in all mesenchymal cells, but the resulting pathology was restricted to posterior cataracts in the lens fibre cells (Bornheim *et al.*, 2008). Later in a screen of 90 human cataract patients, an autosomal mutation in the vimentin gene with similar phenotype was identified in one of the patients (Muller *et al.*, 2009). In conclusion, vimentin plays diverse roles during development, but is not crucially indispensable for embryogenesis.

2.2.3.1. Vimentin phosphorylation

Vimentin fibrils are continuously assembled in a highly organised, dynamic manner and regulated by a complex phosphorylation pattern. The phosphorylation sites of vimentin are under control of several kinases and affect novel cellular functions and protein-protein interactions of vimentin in addition to regulating vimentin dynamics. A long list of kinases interacting with IFs *in vitro* exists. Protein kinase A (PKA) and different PKC isoforms were among the first ones established to phosphorylate vimentin *in vivo* (Eriksson *et al.*, 2004). As discussed earlier, PKCs are also linked to integrin-mediated adhesion and integrin recycling (Woods *et al.*, 2004) and thus, directional cell motility (Parsons *et al.*, 2002). So far, the possible relations of PKCs, integrins, and vimentin in the regulation of intracellular traffic and cell migration have not been studied. In smooth mucle cells, the Rho GTPase effector PAK regulates phosphorylation of vimentin at serine-56. Subsequent disassembly of vimentin filaments stimulates contractility (Tang *et al.*, 2005; Li *et al.*, 2006). In addition, growth factor EGF and PDGF stimulation in endothelial cells and fibroblasts may activate the small GTPases Rac1 and Cdc42 and subsequently spatially reorganise vimentin filaments (Nobes *et al.*, 1995; Valgeirsdottir *et al.*, 1998; Meriane *et al.*, 2000). In addition to cytoplasmic interactions, several mitotic kinases such as cyclin-dependent kinase 1 (Cdk1), Polo-like kinase 1 (Plk1), and Aurora-B have been shown to phosphorylate vimentin in a spatio-temporal manner, suggesting a role for vimentin in cell division. In the regulation of IFs, phosphatases function in equilibrium with kinases. Together they reversibly and continuously conduct phosphorylation and dephosphorylation, regulating the assembly of soluble vimentin subunits to polymers and their disassembly to respond to the needs of cellular changes (Eriksson *et al.*, 2004; Sihag *et al.*, 2007).

2.2.3.2. Vimentin as a signalling scaffold

Several studies have suggested that IFs may act as signalling platforms and scaffolds for signalling molecules. Vimentin can affect the activity and organise the localisation of cell surface molecules. The phosphorylation sites identified in different parts of

vimentin offer a mechanism for vimentin to function as a signalling scaffold (Ivaska *et al.*, 2007). The 14-3-3 intracellular regulatory proteins are major modulators of various cellular functions such as cell cycle and signal transduction through phosphorylation-dependent binding to a multiplicity of signalling molecules. 14-3-3 also interacts with phosphorylated vimentin, but in this case it is vimentin and not 14-3-3 that affects signalling. Vimentin binding limits the availability of 14-3-3 to other target proteins *in vitro* and *in vivo*, thus modulating signalling cascades requiring 14-3-3 (Tzivion *et al.*, 2000). The activated Rho effector ROCKα phosphorylates vimentin and induces filament collapse. As a consequence, ROCKα is released and translocated to the cell periphery. Vimentin is thus linked to ROCKα-mediated signalling and the regulation of actin dynamics (Sin *et al.*, 1998; Eriksson *et al.*, 2009). In injured nerve, vimentin functions as a long-distance messenger. Soluble vimentin subunits bind phosphorylated ERK and directly interact with the nuclear transport factor importin β. By steric hindrance, the complex may protect pERK from phosphatases and thus enable its translocation from the injured axonal site to the cell body for downstream targets (Perlson *et al.*, 2005). These examples consistently elucidate the role of vimentin as a scaffold that regulates the activity of signalling molecules by governing their intracellular distribution and by physically protecting their phosphorylation sites (Eriksson *et al.*, 2009).

In addition to cytoplasmic proteins, a role for vimentin in the regulation of transcription factors has been suggested, as it can interact and sequester transcriptional determinants such as the tumour suppressor p53 (Yang *et al.*, 2005). Very recently, vimentin was evidenced to regulate the transcription of p21 Cdk inhibitor involved in cell cycle progression. Low p21 levels in neuroblastoma patients were linked with poor prognosis, and vimentin expression strongly correlated with p21 both on mRNA and protein level in human neuroblastoma cell lines and in patient tumours. The mechanism by which vimentin regulates p21 promoter activity remains unclear (Mergui *et al.*, 2010).

2.2.3.3. Vimentin-dependent regulation of cell polarity and migration

Cell polarisation is characterised by asymmetrical component distribution to different regions within the cell. Deregulation of polarity is central in EMT and human diseases, particularly in the early stages of cancer (Wodarz and Nathke, 2007). Generation of polarity is fundamental for the apical-basal organisation of epithelial cells. Other variations of cells to display asymmetry include the anterior-posterior polarity observed during cell migration and during cell-cell and cell-ECM adhesion as well as the asymmetry propagated through cell division (Banks and Humbert, 2008). The cell polarity complex that includes Scribble polarity protein is required for directional cell migration. Vimentin has been shown to regulate polarity by stabilising Scribble and protecting it from proteasomal degradation. Scribble binds to vimentin via its PDZ domains and redistributes from IFs to the plasma membrane, thus providing a mechanism for vimentin to promote directional migration (Phua *et al.*, 2009).

A role for vimentin in cell adhesion and migration has been established. Vimentin directly or indirectly (via linker proteins) associates with integrins and the plasma membrane during cell-matrix interactions. The structures where vimentin and actin associate with integrins are termed VAMs. Some proteins like vinculin and plectin are recruited in VAMs, but focal adhesion-targeted signalling proteins have not been observed in VAMs. In the regulation of cell-cell contacts vimentin plays a role particularly in endothelial cells (Ivaska *et al.*, 2007). Cell motility and collagen matrix contraction of vimentin knockout fibroblasts are impaired (Eckes *et al.*, 1998). In scratch-wound assays, MCF10A cells at the edge of the wound upregulate vimentin expression (Gilles *et al.*, 1999) and wound healing in vimentin knockout mice is impaired (Eckes *et al.*, 2000). Vimentin also plays a major role in immune cell migration. It is essential for leukocyte migration both during their adhesion to the vascular endothelium and in the transcellular migration through endothelial cells (Nieminen *et al.*, 2006).

During EMT, vimentin expression is upregulated and the expression of differentiation-specific keratins is downregulated. Keratins are the major IF proteins in epithelial cells, profiles of different keratins expressed varying between cell types. Keratins function as dynamic, regulatory scaffolds controlled by phosphorylation and molecular associations (Magin *et al.*, 2007). Cells derived from advanced stage squamous carcinoma or metastatic head and neck cancer display the classical features of EMT: upregulated vimentin and downregulated keratin expression. Vimentin was further induced by EGF or TGF-β stimulation, together with enhanced motility. Interestingly, siRNA-mediated silencing of vimentin reduced cell proliferation, migration, and invasion, but it also resulted in the re-expression of differentiation-specific keratins (Paccione *et al.*, 2008).

2.2.4. Receptor tyrosine kinases (RTKs) and cancer

Growth factors supervise a range of cellular processes via stimulating the transmembrane RTKs. Extracellular regions correspond to the RTK ligand diversity whereas the intracellular tyrosine kinase domain is highly conserved. In normal cells, RTK activity is tightly controlled and of fundamental importance whereas mutations or alterations in the RTK structure transform them to potent oncoproteins linked to the development and progression of a multiplicity of human cancers (Gschwind *et al.*, 2004). The discovery of the capability of Src to phosphorylate tyrosine residues (Hunter and Sefton, 1980) led to the functional identification of several other RTKs, such as the receptors for EGF (Gullick *et al.*, 1985), VEGF, PDGF, and HGF (ligand for the Met receptor). EGFR (ErbB1, HER1) plays a role in many signalling pathways regulating the growth of both normal and malignant cells; VEGFR is involved in vasculogenesis and angiogenesis. Soon, the oncogene v-ErbB of the avian erythroblastosis virus was established as a variant of the ErbB family member EGFR, connecting an animal oncogene with a human gene responsible for cell growth (Gullick *et al.*, 1985). In kidney epithelial cells, several RTKs were demonstrated to induce

scattering and increased motility whereas only Met promoted branching morphogenesis in 3D matrices, which mimics the formation of tubular epithelia in development (Sachs *et al.*, 1996).

Amplification or overexpression of RTKs is detected in many tumour types, and RTK dysfunction plays key roles in human cancers. Thereby, RTKs are promising therapeutic targets in cancer. Extensive drug design has yielded in novel treatments and many target-specific cancer therapies exist already. The gene encoding ErbB2, *HER2*, is amplified in 30% of invasive breast cancers, correlating with poor clinical prognosis (Slamon *et al.*, 1987). The monoclonal antibody trastuzumab (Herceptin) binds ErbB2 on tumour cell surface and is used as a treatment of *HER2* overexpressing metastatic breast cancer (Pegram *et al.*, 1998). Patients with colorectal cancer are treated with the monoclonal anti-EGFR antibody cetuximab (Erbitux) (Sato *et al.*, 1983); non-small-cell lung carcinoma patients may be treated with gefitinib (Iressa), a small-molecule inhibitor that selectively inhibits EGFR tyrosine kinase activity (Wakeling *et al.*, 1996). Imatinib (Glivec, Gleevec) is a PKC inhibitor derivative that inhibits the activity of the non-RTK Bcr-Abl oncoprotein expressed as constitutively active in 95% of patients with chronic myelogenous leukaemia (CML) (Druker *et al.*, 1996). Imatinib also inhibits the RTKs PDGFR and Kit (Buchdunger *et al.*, 2000) frequently mutationally activated in gastrointestinal stromal tumours (GISTs). At present, imatinib is approved for the treatment of CML, advanced GIST, and various other malignancies. Moreover, therapeutic targeting of VEGF and VEGFR signalling to inhibit angiogenesis is another approach to prevent tumour progression. Bevacizumab (Avastin) anti-VEGF antibody (Presta *et al.*, 1997) is the first drug inhibiting angiogenesis. It is used in the treatment of colorectal cancer, non-small-cell lung carcinoma, and breast cancer. Expectations regarding RTK-based treatments are high as several promising compounds are in clinical trials and other strategies to treat cancer are being extensively investigated (Gschwind *et al.*, 2004).

2.2.4.1. The Axl RTK subfamily

The Axl subfamily of mammalian RTKs consists of Tyro3 (or Sky), Axl (or Ufo), and Mer, the TAM receptors. They possess distinct expression profiles and functions, but share the vitamin K-dependent and secreted ligand, growth arrest-specific gene 6 (Gas6) (Hafizi and Dahlback, 2006). Tyro3 and Mer also bind another ligand, blood coagulation regulator protein S (Dahlback and Villoutreix, 2005). Gas6 promotes growth and metastasis in experimental cancer models, and induces mitosis in tumour cells. Circulating leukocytes produce minimal Gas6, but malignant cells promote tumour growth by deluding infiltrating leukocytes to upregulate Gas6. High level coexpression of Gas6 and Axl is frequent in human glioma tumours and correlates with poor cancer patient survival (Hutterer *et al.*, 2008). Also, upregulated Gas6 and Axl coexpression has been reported in endometrial (Sun *et al.*, 2003), ovarian (Sun *et al.*, 2004), and gastric cancer (Sawabu *et al.*, 2007) as well as in patients around Chernobyl with thyroid tumours (Ito *et al.*, 2002). Axl was originally identified in chronic myelogenous leukemia patients due to its *in vitro* transforming activity (O'Bryan *et al.*,

1991; Janssen *et al.*, 1991). In adult organs and cells Axl is ubiquitously expressed whereas Tyro3 is predominantly expressed in the central nervous system and brain (Ohashi *et al.*, 1994) and in bone osteoclasts (Nakamura *et al.*, 1998). Mer is expressed in monocytes and tissues of epithelial and reproductive origin (Graham *et al.*, 1994) and it mediates phagocytosis of apoptotic cells by macrophages either via an αvβ5-dependent and FAK-involving mechanism or via the GEF Vav, both pathways converging on Rac GTPase (Scott *et al.*, 2001). The Axl RTK subfamily members each comprise two N-terminal immunoglobulin (Ig)-like domains and two FN type III repeats in the extracellular regions, a single-pass transmembrane domain, and an intracellular tyrosine kinase domain with intrinsic activity. In the case of Axl, the kinase domain can be activated by Gas6 ligand binding to the Ig-like domains (Varnum *et al.*, 1995). Subsequently the receptor dimerises, forming either homo- or heterodimers. Tyro3 dimerisation hinders Gas6 from binding to its major binding site. Thus, a possibility of ligand-independent dimerisation and activation exists also for Axl (Heiring *et al.*, 2004).

Mice lacking any single receptor of the TAM family are viable, fertile, and display no gross anatomical defects. It appears that Tyro3 and Axl are dispensable during embryonic development (Lu *et al.*, 1999). Mer knockout induces an autoimmune phenotype, suggesting a role in immune homeostasis (Cohen *et al.*, 2002). The Royal College of Surgeons rat model carries a hereditary defect leading to retinal dystrophy and eventually, blindness. Mutation in the gene encoding Mer was identified behind the phenotype (D'Cruz *et al.*, 2000). RTKs are prone to forming heterodimers; Axl forms even homodimers. Double mutants, Tyro3/Axl and Tyro3/Mer are superficially healthy but compromised with defects in spermatogenesis (Lu *et al.*, 1999). Adult triple mutants are viable and quite normal for the first several weeks, but then develop degenerative phenotypes: multiple major organ defects, neuronal abnormalities, and physiological deficits. In addition, they are blind and produce no mature sperm (Lu *et al.*, 1999). The possibility of receptor redundancy has been under debate because of the RTK convention on heterodimer formation, overlapping binding specificities of the TAM receptors, and co-expression in several cell types (Binder and Kilpatrick, 2009). Furthermore, the autoimmunity phenotype exhibited by mice deficient in a single TAM receptor is far milder compared to the severe phenotype in triple knockout mice (Lu and Lemke, 2001).

2.2.4.2. The multiple interactions of Axl

Axl overexpression is implicated in a variety of human cancers. *In vivo*, Axl is essential for tumour growth, invasiveness, and metastasis (Gjerdrum *et al.*, 2010). In prostate cells, Axl expression correlates with the metastatic potential (Jacob *et al.*, 1999). 10-fold higher Axl expression was reported in colon carcinoma metastases compared to other normal or malignant tissues (Craven *et al.*, 1995). Axl is overexpressed in a subgroup of breast cancer (Meric *et al.*, 2002). Additionally, Axl is highly expressed in leukemia (Neubauer *et al.*, 1994) as well as in human tumour cells *in vitro*. It modulates a variety of endothelial cell processes including proliferation,

migration, survival, angiogenesis (Holland *et al.*, 2005; Li *et al.*, 2009), and growth of breast carcinoma and glioma cells (Holland *et al.*, 2005; Vajkoczy *et al.*, 2006). Axl inhibition reduces lung (Li *et al.*, 2009) and breast cancer (Holland *et al.*, 2005; Li *et al.*, 2009) xenograft tumour growth and prevents breast cancer cell migration and metastasis to the lung *in vivo* (Li *et al.*, 2009). Expression of regulatory transcription factors is a common feature of malignant cancers. Precisely, introduction of transcription factors Twist, Zeb2, Snail, or Slug in breast epithelial MCF10A cells induces EMT: cells lose epithelial markers E-cadherin and β-catenin, acquire mesenchymal morphology and increased expression of mesenchymal markers (vimentin, N-cadherin), as well as show upregulation of Axl (Gjerdrum *et al.*, 2010). Futhermore, Axl regulates EMT-induced breast cancer metastasis and predicts poor patient survival independent of other EMT markers such as E-cadherin (Gjerdrum *et al.*, 2010). In accordance, a selective small-molecule inhibitor of Axl, R428, was recently published to inhibit angiogenesis in tumour models. Also, orally administered R428 reduced metastasis and extended survival in breast cancer mouse models. Specifically, R428 was shown to dose-depently reduce the expression of Snail (Holland *et al.*, 2010). Interestingly, increased Axl expression was identified as a mechanism of certain type of breast cancer cells to acquire resistance to a cancer drug, lapatinib. Sensitivity of these cells on lapatinib could be restored using a potent multikinase inhibitor GSK1363089 (foretinib), which inhibits HGF and VEGF RTKs in addition to Axl (Liu *et al.*, 2009). The potential of GSK1363089 as a novel cancer drug is currently investigated in phase II studies directed by the healthcare company Glaxo Smith Kline.

Several binding partners for the Axl intracellular domain (ICD) have been defined. First, in an expression library screen, putative substrates and their individual docking sites within the Axl ICD were identified (Braunger *et al.*, 1997). The discovered interacting substrates include PI3K regulatory subunits p85α, p85β, PI-specific enzyme PLCγ that is strongly activated by all families of GFRs and important for subsequent cell motility, growth factor receptor-bound protein 2 (Grb2) adaptor protein involved in intracellular signal transduction, Src tyrosine kinase, and leukocyte-specific protein tyrosine kinase (Lck). Later on, a yeast-two hybrid screen on a human heart cDNA library confirmed some of the interactions and revealed several new, including one more PI3K subunit, p55γ, and a novel phosphatase and tensin homologue (PTEN)-like phosphatidyl inositol (PI) phosphatase, C1 domain-containing PTEN (C1-TEN) (Hafizi *et al.*, 2002), which inhibits cell survival, proliferation, migration, and specifically the PI3K/Akt pathway (Hafizi *et al.*, 2002; Hafizi *et al.*, 2005). Interestingly, the Axl ICD itself was among the Axl-interacting proteins, suggesting that homodimerisation may be important for signalling. Collectively, most of the identified binding partners contain phosphotyrosine-binding Src homology 2 (SH2) domains, possibly responsible for the interactions. Activation of PI3K leads to Akt (also called protein kinase B) phosphorylation and nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) activation. These seem critical events in Axl signalling, transducing the survival signal and prevention of apoptosis. The interactions with different PI3K isoforms and Grb2 offer specific mechanisms for affecting the

signalling pathways downstream of Akt. Binding of Grb2 alternatively of the Akt survival pathway can engage the ERK signalling pathway responsible for the effects of Axl signalling on proliferation. Roles in inflammation and vascular remodelling have been suggested (Binder and Kilpatrick, 2009; Lemke and Rothlin, 2008). Taken together, Axl plays a multiformity of regulatory roles in cancer cell signalling (Figure 8).

The autoimmunity phenotype of Axl knockout mice suggests that Axl is important not only during oncogenesis, but also immunogenically. Natural killer (NK) cells belong to the army of innate immunity. The function of this system is to recognise and destroy pathogen-infected cells. Axl regulates the activity of NK cells in concert with cytokine receptors; NK cells isolated from the triple TAM receptor knockout mice have poor cytotoxic activity and all of the three receptors are essential for NK cell maturation and function (Caraux *et al.*, 2006). Interleukin (IL)-15 plays a critical role in the maturation of NK cells required for target-cell recognition and killing. IL-15 also (Lu and Lemke, 2001) protects cells from apoptosis. Unexpectedly, Axl associates and cooperates with the IL-15 receptor α (IL-15Rα) (Figure 8). IL-15 (but not IL-2 or other cytokines) transactivates Axl: it induces a rapid phosphorylation of Axl even in the absence of Gas6 (Budagian *et al.*, 2005). Moreover, Ebola virus has been shown to use the physiological functions of Axl for cell entry (Shimojima *et al.*, 2007).

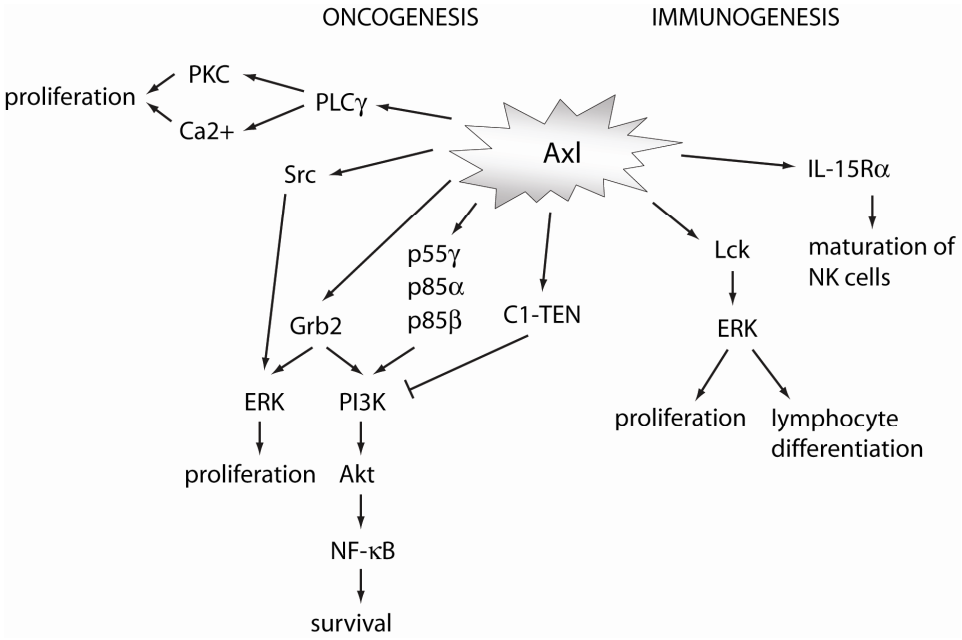

Figure 8. Axl interactors and signalling. The schematic summary demonstrates some of the known Axl signalling and interaction partners.

3. AIMS OF THE STUDY

The integrin family interacts with the ECM to confer adhesive and motile properties on cells. $\alpha 2\beta 1$ integrin plays the major part in adhesion and spreading on collagen type I, but additional receptors are required. Syndecans are cell surface heparan sulphate proteoglycans that appear to have a role in adhesion to many ECM components. However, little is known about their role in adhesion on collagen. Vimentin is an important marker of EMT. Also, vimentin expression is related with a highly motile and invasive phenotype. All these cellular molecules: integrins, syndecans, MMPs, and vimentin are very important for adhesion and motility and therefore cancer. The objective of this study is to characterise the collaboration of vimentin, different kinases, integrins, and their co-receptors in cancer cell adhesion, invasion, and metastasis.

The specific aims of this study were:

- To study the role of vimentin in the regulation of integrin traffic and cell motility.
- To clarify the role of vimentin in EMT and in regulating the expression of migratory genes.
- To elucidate how syndecans and integrins together regulate cell adhesion, organisation of the actin cytoskeleton, matrix remodelling, and invasion.

4. MATERIALS AND METHODS

More detailed information on methods and reagents is available in the original publications (I-IV).

Primary antibodies

Antigen	Antibody and supplier	Application	Used in
β1 integrin	AIIB2, Santa Cruz	ELISA	I
PKCε	antiserum 130, (Yasui *et al.*, 2001)	IF, WB, EM	I
phospho-serine (vimentin)	Schaap and Parker, 1990	WB	
vimentin	anti-human mAb IgG, Santa Cruz	IF, WB	I
vimentin	pAb, Santa Cruz	EM	
vimentin	anti-mouse mAb 40E-C, Santa Cruz	IF, WB	I
myosin heavy chain II A/B	PRB-440P/PRB-445P, Covance	WB	I
phospho-serine	Sigma-Aldrich	WB	I
α-tubulin	12G10, Hybridoma Bank	WB	I, II, IV
α2 integrin	MCA2025, AbD Serotec	FACS, IF, WB	I, III, IV
β-actin	ab8226, Abcam	WB	II
Hsc70	SPA-815F, Stressgen Bioreagents	WB	II
β-catenin	ab32572, Abcam	WB	II
E-cadherin	#4065, Cell Signaling Tech.	WB	II
E-cadherin	ab1416, Abcam	IF	II
N-cadherin	ab18203, Abcam	WB	II
human HLA	Hb116, (Koskinen *et al.*, 2004)	IF	II
Axl	AF154, R&D Systems	IF, WB	II
Axl	H-124/SC-20741, Santa Cruz	IF, WB	II
Slug	L40Cb #9585S, Cell Signaling	IF, WB	II
H-Ras	MCA2884, AbD Serotec	WB	II
α2 integrin	1936, Chemicon	IF	III
α2 integrin	12F1, BD Pharmingen	FACS	III
α2 integrin	16B4, AbD Serotec	FACS	III
α2 integrin	P1H5, Santa Cruz	FB	III, IV
α5 integrin	MCA1187, AbD Serotec	FACS	III
b1 integrin	P5D2, Hybridoma Bank	FACS	III
syndecan-1	B-B4, AbD Serotec	FACS, IF	III, IV
syndecan-2	M-140, Santa Cruz	FACS	III

syndecan-4	AF2918, R&D Systems	FACS, IF	III, IV
K-Ras	SC-309, Santa Cruz	WB	IV
MT1-MMP	LEM-2/15.8, Chemicon	FACS	IV
phospho-MLC	ab4720, Abcam	IF, WB	IV

*Abbreviations: EM=electron microscopy, FACS=flow cytometry, FB=function blocking, IF=immunofluorescence, WB=Western blotting

Expression vectors

The following expression vectors were generated:

I: pCMV-vim (S4,6,7,8,9A), pCMV-vim (S4,6,7,8,9D)

III: pEGFP-α2 (Pellinen *et al.*, 2006), pEGFP-SS-α2mat, pEGFP-Sdc2 (syndecan=Sdc), pMMP1-luc

Expression vectors with the following inserts were kindly provided:

I, III, IV: pAWneo2-α2 (Ivaska *et al.*, 1999)

II: Axl, pIRES-EGFP-Axl, pIRES-EGFP-Slug (Prof. J. Lorens, University of Bergen)

III, IV: novo-pEGFP-N2-Sdc1, pEGFP-Sdc4, pcDNA-Sdc1, and pcDNA-Sdc4 (Dr M. Tuittila, University of Turku), pcDNA-Sdc1-core (Prof. R. Sanderson, University of Arkansas)

Cell lines

Cell line	Species	Cell type / organ of origin	Used in
MEF	mouse	embryonic fibroblast	I
Saos	human	non-transformed osteosarcoma	I
MCF10A	human	mammary epithelial	II
SW13	human	adrenal carcinoma	II
MDA-MB-231	human	breast adenocarcinoma	II, III, IV
CHO	hamster	ovary	III, IV

The following stable cell lines were generated:

I: Saos-EGFP-α2

III, IV: CHOwtα2, CHO745α2, MDA-MB-231: GFP, GFPSdc1, GFPSdc2, GFPSdc4

The following cell lines were kindly provided:

II: MDA-MB-231: shLuc, shAxl, IRES-GFP, IRES-GFP-Axl; MCF10A-Hras, MCF10A-Slug (Prof. J. Lorens, University of Bergen)

Reagents

Reagent	Application	Supplier	Used in
BIM I GF109203X	PKC inhibition	Cayman Chemical	I
transferrin	immunofluorescence	Molecular Probes	I
fibronectin	coating	Sigma-Aldrich	I
Fugene 6	transfection	Roche	I
QuikChange® XL Kit	site-directed mutagenesis	Stratagene	I
collagen I	plate coating	Sigma-Aldrich	I, III
Lipofectamine 2000	transfection	Invitrogen	I, II, III, IV
mowiol	immunofluorescence	Calbiochem	II, III, IV
phalloidin	immunofluorescence	Molecular Probes	II, III, IV
DABCO	immunofluorescence	Sigma-Aldrich	II, III, IV
RT-PCR primers, probes	RT-PCR and TaqMan qRT-PCR	Roche	II
Cell Trackers	live cell staining	Invitrogen	II
collagenase XI	release of cells from tissue	Sigma-Aldrich	II
DAPI	immunofluorescence	Sigma-Aldrich	III
propidium iodide	adhesion assay, flow cytometry	Sigma-Aldrich	III
WST-1	proliferation assay	Roche	III
Rhotekin-RBD pulldown kit	Rho activation assay	Cytoskeleton	IV
pepsin-extracted collagen	coating, gel contraction, invasion	Nutacon	IV
rat tail collagen	coating, gel contraction, invasion	Sigma-Aldrich	IV
collagenases IA and IV	release of cells from 3D collagen	Sigma-Aldrich	IV
Y27632	ROCK inhibition	Sigma-Aldrich	IV
GM6001	MMP inhibition	Calbiochem	IV
hematoxylin and eosin	tissue staining	Sigma-Aldrich	IV

siRNAs

Target	Name/cat. no. of siRNA or publication	Supplier	Used in
scrambled ctrl	All Stars negative control	Qiagen	II, III, IV
syndecan-1	Beauvais *et al.*, 2004	Qiagen	III, IV
syndecan-4	Rauch *et al.*, 2005	Qiagen	III, IV
KRAS_1	#SI02662051	Qiagen	IV
KRAS_2	#4390824	Ambion	IV
vimentin_A	M-003551-01-005	Dharmacon	II
vimentin_B	#SI00302190	Qiagen	II

vimentin_C	#SI002655198	Qiagen	II
Axl_A	sc-29769	Santa Cruz	II
Axl_B	sc-44285	Santa Cruz	II

Methodology

Method	Used in
Integrin internalisation and recycling assay	I
Transwell migration assay	I
2D electrophoresis	I
Cellular fractionation	I
Electron microscopy	I
Cell culture	I, II, III, IV
Statistical analysis	I, II, III, IV
Transfection and generation of stable cell lines	I, II, III, IV
Immunofluorescence microscopy	I, II, III, IV
Western blot analysis	I, II, IV
Site-directed mutagenesis	I, III
RNA extraction	II
Lung extravasation assay	II
Illumina cDNA microarray	II
Time-lapse microscopy	II
Flow cytometry	II, III, IV
Proliferation assay	II, III, IV
Analysis of mRNA expression by RT-PCR and TaqMan qRT-PCR	II, III, IV
Adhesion assay	III, IV
Scratch-wound migration assay	III
Collagen invasion assay	IV
Floating collagen matrix contraction assay	IV

Animals

Mice: Female athymic nude mice (Hsd: Athymic Nude-nu; Harlan Scandinavia, Allerod, Denmark), aged between 4-6 weeks.

5. RESULTS

5.1. PKCε-MEDIATED PHOSPHORYLATION OF VIMENTIN CONTROLS INTEGRIN RECYCLING AND MOTILITY (I)

5.1.1. Vimentin is a PKC substrate in vesicles

A novel PKC isoform, PKCε, has been shown to control an unidentified step that regulates integrin recycling in motile cells (Ivaska *et al.*, 2002). To identify the substrates of PKCε controlling the liberation of β1 integrin-containing vesicles, a release assay utilising biotinylation was performed on vesicles derived from PKCε reconstituted (PKCεRE) MEFs treated with the specific, cell-permeable PKC inhibitor BIM I. Myosin heavy chain type II A/B (MHCIIA/B) and vimentin were detected by mass spectrometry fingerprinting as kinase substrates (I, Fig. 1A). The presence of these putative PKCε substrates in the same subcellular compartment with PKCε and β1 integrin was determined by sucrose gradient fractionation. It has been shown, that BIM I-induced vesicular accumulation shifts PKCε and β1 integrin localisation to fractions 7-9 (Ivaska *et al.*, 2002). Similarly, BIM I treatment shifted vimentin from a cytosolic compartment (fractions 1-4) to a denser, PKCε-positive one (fractions 7-9) (I, Fig. 1B). Also, MHCIIA/B was shifted to a denser fraction, but the overlap with PKCε was only partial. In immunofluorescence stainings, PKC inhibition had no effect on the localisation of MHCIIA or B (data not shown) and they were not studied further. In contrast, the IF-like structures recognised as vimentin became fragmented and patchy on PKC inhibition (I, Fig. 1C). PKCε and vimentin staining patterns partially overlapped. Fractionated, isolated vesicles were further analysed by electron microscopy and immunogold labelling. Again, PKCε and vimentin colocalised in a vesicular comparment in extracts from BIM I-treated cells (I, Fig. 1D). These data recognise vimentin as a PKC substrate in vesicles containing β1 integrin and PKCε.

5.1.2. PKCε mediates phosphorylation of vimentin

Next, the dependence of vimentin phosphorylation on PKCε was examined in intact cells to exclude the role of other BIM I sensitive kinases possibly associated with the vesicles. PKCε knockout and PKCεRE MEFs were control- and BIM I-treated and the whole-cell extracts were separated by 2D electrophoresis. Vimentin and serine phosphorylation were detected with Western blotting, indicating a higher level of vimentin phosphorylation in PKCεRE compared to PKCε knockout cells (I, Fig. 2A). Phosphorylated vimentin was lost on PKC inhibition. Also, PKC inhibition reduced overall serine phosphorylation (I, Fig. 2A). These data suggest that vimentin is phosphorylated by PKC. To clarify the possible role of vimentin in PKCε and β1 integrin getting trapped in the vesicular compartment upon PKC inhibition, the dissociation of vimentin and PKCε from vesicles was investigated. Fractionation of

biotinylated vesicles on a sucrose gradient illustrated the dissociation of vimentin from the vesicles similarly to PKCε in presence of cytosol and energy (I, Fig. 2B). Furthermore, they were shown to dissociate as a complex by immunoprecipitation of vimentin from soluble fraction after letting PKCε and vimentin detach from isolated vesicles as at least half of the released PKCε had been released as a complex with vimentin (I, Fig. 2C). Finally, the soluble and membrane-bound fractions were separated using centrifugation to determine the site of vimentin phosphorylation and the relative amounts of phosphorylated vimentin in the fractions. Western blotting with all available phospho-vimentin specific antibodies (Ser6, Ser33, Ser38, Ser50, Ser55, Ser71, Ser82) evidenced that the released vimentin largely contained the phospho-Ser6 epitope and some Ser33 (I, Fig. 2D-F). In summary, the phosphorylation of vimenin Ser6 and possibly other serine residues may support the dissociation of the vimentin-PKCε complex.

5.1.3. Vimentin induces cell motility in a PKCε-dependent manner

In line with earlier experience on vimentin-induced augment in migration during epithelial cell transformation (Hendrix *et al.*, 1996), expression of vimentin in vimentin-negative epithelial MCF7 cells increased cell motility (I, Fig. 3A). In order to study the importance of vimentin for PKCε-controlled cell motility, vimentin was expressed in PKCε knockout and PKCεRE cells. Haptotactic migration towards fibronectin was higher in PKCεRE cells compared to the null cells and further increased when vimentin was introduced whereas vimentin expression had no effect on the migration of PKCε knockout cells (I, Fig. 3B). Reported PKC sites of vimentin were mutated to alanine to study the role of these sites on vimentin-induced cell motility. Vimentin mutants were expressed in PKCεRE cells and detected with a monoclonal antibody that does not recognise murine vimentin. Consequently, vimentinS4,6,7,8,9A showed a fragmented localisation (I, Fig. 3C) resembling that of wildtype (wt) vimentin after BIM I-treatment (I, Fig. 1C). Endogenous vimentin paired with the introduced wt vimentin forming mixed IF-like filaments. Mutant vimentinS4,6,7,8,9A also paired with endogenous vimentin (I, Fig. 3D) whereas the localisation of another mutant vimentinS6,33,38,50,71,82A was diffuse with only few filaments (not shown). VimentinS4,6,7,8,9A expression in PKCε knockout and PKCεRE cells had no effect on their motility, while vimentinS6,33,38,50,71,82A induced a slight, yet statistically non-significant increase (I, Fig. 3E). The five N-terminal serines were substituted with negatively charged residues to further inspect their input. Expression of this vimentinS4,6,7,8,9D in PKCε knockout cells rescued the defect in cell migration (I, Fig. 3F), confirming the importance of the phosphorylation of these residues of vimentin in PKCε-dependent cell motility.

5.1.4. Phosphorylation of vimentin is essential for integrin localisation and motility

Eventually, the significance of vimentin phosphorylation in the localisation of integrins and PKCε was elucidated. Expression of vimentinS4,6,7,8,9A induced the accumulation of PKCε in vesicles (I, Fig. 4A, top panels) from the normal, cytosolic localisation detected upon wt vimentin expression (I, Fig. 4A, bottom panels). In contrast, vimentinS4,6,7,8,9D recruited PKCε to filaments (I, Fig. 4A, middle panels). MEF cells of this study lack endogenous collagen-binding integrins. In cells, GFP-α2 integrin forms a functional heterodimer with β1 integrin. GFP-α2 was expressed in MEF cells spreading on collagen upon PKC inhibition. Suggesting that it functions similarly to endogenous β1 integrin, GFP-α2 was trapped in a vesicular compartment with PKCε upon PKC inhibition (I, Fig. 4B). The expression of the mutant vimentinS4,6,7,8,9A induced the accumulation of GFP-α2 integrin in vesicles in a manner similar to PKC inhibition (I, Fig. 4B). Furthermore, vimentinS4,6,7,8,9A expression reduced recycling of both α2 and β1 integrin (I, Fig. 4C), affecting the endocytosed pool of integrin. Importantly, the effect is not general as transferrin endocytosis was unaffected (I, Fig. S3A, B, 4D). Integrin traffic was illustrated in human osteosarcoma Saos-2 cells visualising the dynamics of GFP-α2 in live cells. In these cells, GFP-α2 mainly localised at the plasma membrane, integrin vesicles moving along cytoplasmic filaments towards the cell surface (I, Fig. 5; Supplementary video 1). On PKC inhibition, α2 integrin appeared in static intracellular vesicles with minor movement towards the membrane (I, Supplementary video 2). In conclusion, these data present that PKCε-mediated phosphorylation of vimentin is required for the integrin/PKCε-containing vesicles to be released from the association with vimentin (I, Fig. 6).

5.2. VIMENTIN REGULATES EMT INDUCTION AND MIGRATION THROUGH AXL (II)

5.2.1. Vimentin is induced in migrating cells

Vimentin expression has been shown to be induced in migrating cells at the wound edge in MCF10A human mammary epithelial cells (Gilles *et al.*, 1999). In accordance, upon scratch-wounding of a confluent monolayer of MDA-MB-231 human breast adenocarcinoma or MCF10A cells, we show that both vimentin (II, Fig. 1a) and Axl (II, Fig. 4e) were upregulated in cells at the wound edge. Supporting the theory of reversible EMT, cells at the wound edge showed additional alterations typical for EMT in their phenotype, namely loss of E-cadherin and upregulation of actin stress fibres (II, Fig. S1). Basal levels of vimentin were higher in MDA-MB-231 (II, Fig. 1a) and they migrated significantly more compared to MCF10A cells in a scratch-wound assay (II, Fig. 1b). When efficiently silencing vimentin using siRNA (II, Fig. 1c), migration was reduced in both cell lines (II, Fig. 1d, e). Next, EMT was induced in MCF10A cells by ectopic expression of transcription factor Slug or oncogenic Ras-V12. Consequently,

epithelial markers E-cadherin and β-catenin were downregulated while EMT markers vimentin and N-cadherin were upregulated (II, Fig. 2a, b). Cells expressing Slug showed a distinct, EMT-like morphology with long and thin protrusions (II, Fig. 2c). Furthermore, silencing of vimentin in these cells returned the morphology of Slug-expressing cells to resemble the rounder-shaped control GFP cells (II, Fig. 2c), evidencing the vimentin-dependency of the phenotype. Also typically for EMT, the motility of both cell lines MCF10A-Slug and MCF10A-Ras was induced compared to the control GFP cells (II, Fig. 2d, e). Upon vimentin silencing, migration was dramatically abolished in all the experimented cell lines (II, Fig. 2d, f). In summary, vimentin is upregulated with with other EMT markers in response to wounding and Slug and H-Ras expression.

5.2.2. Vimentin regulates migratory genes

To assess the role of vimentin in EMT, the effects of vimentin silencing on the expression of other genes were analysed in a cDNA microarray screen in MCF10A cells. In total, 85 genes were significantly upregulated and 35 downregulated. 11 of these were chosen for further studies (II, Fig. 3a; red=upregulation, blue=downregulation) and *in vivo* exploration of the results. Both the microarray and quantitative PCR data analyses showed downregulation of *AXL*, *PLAU*, and *ITGB4* that are linked with a migratory phenotype as well as upregulation of charasteristics of normal epithelium, *RAB25*, *EHF,* and *KLK5* (II, Fig. 3a, b). Then, the expression of vimentin and the correlative genes in other cell lines and patients was investigated in a study integrating multiple gene profiling studies consisting of 733 breast cancer patient samples, samples from 179 person with normal breast tissues, and 92 breast cancer cell lines. Importantly, vimentin correlated positively with the expression of *AXL*, *PLAU*, and *ITGB4* in clinical cancer samples and cell lines (II, Fig. 3c). *AXL* and *PLAU* also correlated with vimentin in normal breast samples (II, Fig. 3c). Vimentin and *RAB25* small GTPase had a strong negative correlation in all the samples. In addition, vimentin and two of the upregulated genes of the cDNA microarray screen, *KLK5* and *KRT6B,* inversely correlated in normal clinical samples, but in cancer samples vimentin and *KRT6B* were positively correlated (II, Fig. 3c). These data indicate a common value for vimentin in the regulation of migratory genes.

5.2.3. Vimentin rules migration by commanding Axl in Slug-induced EMT

The link between vimentin and the receptor tyrosine kinase Axl implicated in a migratory phenotype (Holland *et al.*, 2005) was chosen for more detailed investigations. MDA-MB-231 cells that express more vimentin and migrate more than MCF10A cells (II, Fig. 1a, b) also express higher level of Axl (II, Fig. 4a, b). Similarly, when EMT is induced in MCF10A cells by Slug or Ras expression, Axl is upregulated (II, Fig. 4c, 4d) in addition to increased vimentin expression (II, Fig. 2a, b, 4c) and migration (II, Fig. 2c, d). In MDA-MB-231 cell immunofluorescence samples, Axl and

vimentin did not colocalise, but in cells at the edge of the scratch-wound they both were upregulated. Furthermore, Axl localised to the lamellipodia which were extended towards the wound area (II, Fig. 4e). Upon acquisition of a migratory, EMT-like phenotype and Axl expression in MCF10A cells at the edge of a monolayer, E-cadherin was lost (II, Fig. 4f). Expression of Slug in MCF10A cells had the same end results: upregulation of Axl and downregulation of E-cadherin (II, Fig. 4f). Migrating cells at the scratch-wound edge showed a 38% upregulation of endogenous Slug with vimentin and Axl (II, Fig. 4g), suggesting a role for Slug in the regulation of Axl by vimentin.

Vimentin was also shown to regulate Axl expression on mRNA level (II, Fig. 2a, b). Moreover, silencing of vimentin reduced Axl levels on protein level in MDA-MB-231 cells (II, Fig. 5a). Three individual siRNAs were tested to exclude the possibility of non-specific effects (II, Fig. S3a). In contrast, silencing of Axl using two different siRNAs had no effect on vimentin levels (II, Fig. 5a, S3a). Furthermore, Axl upregulated by the expression of Slug and Ras in MCF10A cells (II, Fig. 5b, 4c, d) was evidenced to be dependent on vimentin as silencing of vimentin was enough to reduce Axl expression (II, Fig. 5b, c). Silencing of either vimentin or Axl reduced migration in scratch-wound assays with MDA-MB-231 (II, Fig. 6a, S3b) and MCF10A cells (II, Fig. 6b) as well as in time-lapse migration experiments with MCF10A-Ras cells (II, Fig. 6c). The SW13 cell line has two subtypes either expressing vimentin or not (II, Fig.7a). Migration of these SW13vim+ and SW13vim− cells was studied to define the importance of vimentin in Axl-derived cell migration. In SW13vim− cells, expression of Axl had a stronger impact on migration compared to the SW13vim+ cells (II, Fig.7b). Furthermore, in MDA-MB-231 cells shRNA-silenced for Axl, downregulation of vimentin had no further effect on migration in scratch-wound assays (II, Fig.8e) or in time-lapse migration experiments on cell-derived, 3D matrix (II, Fig.8f), suggesting that vimentin regulates migration via control over Axl. Lastly, the importance of Axl for migration *in vivo* was experimented by injecting MDA-MB-231 control and Axl shRNA-silenced cells into the tail vein of mice. Extravasation of the injected human cells to lungs was measured after 48 h. Control cells had extravasated into tissue significantly more compared to Axl-silenced cells (II, Fig. 8g), evidencing the importance of Axl for invasion *in vivo*.

5.3. THE COOPERATION OF SYNDECAN-1 AND INTEGRIN α2β1 IN CELL ADHESION (III)

5.3.1. GAGs are required for cell adhesion on collagen

We hypothesised, that PGs play a role in adhesion to collagen in collaboration with integrins. To first elucidate the possible involvement of GAG chains in adhesion, we began the study using CHO cells. CHO cells have no endogenous collagen-binding integrins and the CHO745 mutant cell line is defective in the biosynthesis of both HS and CS GAG chains (Esko et al., 1985). Neither CHOwt nor the CHO745 cell line

adhered to collagen I whereas both bound to FN in a dose-dependent manner (III, Fig. 1A). When transfected to express α2β1 integrin, CHOwt cells became able to bind to type I collagen (III, Fig. 1A, B), dependent on α2β1 integrin (Fig. S1). Strikingly, CHO745 cells lacking endogenous HS and CS GAG chains were completely unable to adhere to collagen I, even when expressing α2β1 (III, Fig. 1A, B, S1). These results implicate that GAGs are important for cell adhesion to collagen. Interestingly, this requirement seems to apply to other cell types as well as enzymatic disruption of HS and CS from MDA-MB-231 human mammary carcinoma cells results in similar defects in collagen binding (III, Fig. 3A). Furthermore, the role of proteoglycans seems to be critical only to cell adhesion to monomeric collagen since the effects of HS and CS disruption on binding to fibrillar collagen are only moderate (III, Fig. 3B). Interestingly, dramatic changes in the actin cytoskeleton are seen in both CHO cell lines when cells lacking glycosaminoglycan chains are plated on collagen I (III, Fig. 2), suggesting that GAGs are irreplaceable for actin organisation when cells adhere on collagen.

Integrin α2β1 recognises a specific triple helical sequence in type I collagen. Adhesion to the hexapeptide containing this GFOGER sequence is not affected by the absence of HS and CS GAGs (III, Fig. 3C), indicating that cell adhesion to intact collagen requires additional receptors to the integrins. MDA-MB-231 cells spread similarly on the ECM ligand of α2β1 integrin, collagen type I or on the GFOGER hexapeptide, and shared a uniform F-actin cytoskeleton (III, Fig. 3D). These data implicate that adhesion to the integrin recognition site alone mediated by the α2 integrin I-domain is independent of PGs but PGs are essential in adhesion to full length collagen.

5.3.2. Syndecan-1 and α2β1 integrin cooperate in cell adhesion

Syndecans appear to have a role in cell adhesion to many extracellular matrices but their functions in cell-collagen interactions have not been intensively studied. To identify the cooperative PG supporting α2β1 integrin functions, GFP-tagged syndecan constructs were overexpressed in MDA-MB-231 cells that endogenously express syndecans (III, Fig. S2). GFP-syndecan-1 but not GFP-syndecan-2 or -4 induced adhesion to collagen as GFP-syndecan-1 expressing cells adhered 31-52% better on collagen than the control GFP expressing cells (III, Fig. 4A). Adhesion to FN was also slightly intensified in cells expressing GFP-syndecan-1 while GFP-syndecan-2 or -4 expression produced no effect. GFP-syndecan-1 expression induced the expression of the FN-specific integrin α5 and the increase in adhesion was fully dependent on α5 integrin (III, Fig. 4B). α2β1 integrin-mediated adhesion to the GFOGER hexapeptide remained unaltered regardless of any GFP-syndecan-1, -2, or -4 expressed (III, Fig. 4C), supporting our results on PG-independent α2β1 integrin recognition of the GFOGER sequence. Transient expression of non-tagged syndecan-1, -2, and -4 had similar effects on adhesion as the GFP-tagged syndecans: syndecan-1 expression

induced adhesion to collagen and FN (III, Fig. 4D, E). The effects were weak though, explained by low transfection efficiency (III, Fig. S2D).

The interplay between syndecan-1 and α2β1 integrin was further confirmed by syndecan-1 silencing in MDA-MB-231 cells. The siRNA silencing was effective (III, Fig. 5A). Compared to scrambled siRNA-transfected control cells, syndecan-1-silenced cells adhered significantly less on collagen (III, Fig. 5B). Again, only adhesion to full-length collagen was affected and adhesion to GFOGER was not inhibited (III, Fig. 5C). Furthermore, silencing of syndecan-2 and -4 did not reduce adhesion to collagen but somewhat increased adhesion (III, Fig. 5E). To verify the supportive role of syndecan-1 in α2β1 integrin-mediated adhesion, GFP-syndecan-1 was transiently expressed in CHO cells. GFP-syndecan-1 expression in CHOwtα2β1 significantly boosted adhesion whereas in the absence of collagen-binding integrins in CHOwt cells, the expression of GFP-syndecan-1 was not sufficient to promote cell adhesion (III, Fig. 6A). Importantly, anti-syndecan-1 antibody could not prevent collagen adhesion as α2-integrin function-blocking antibody did (III, Fig. 6B), further demonstrating that syndecan-1 alone does not mediate adhesion to collagen. Taken together, these data confirm the role of syndecan-1 in supporting α2β1 integrin-mediated adhesion.

CHO745α2 cells are unable to synthesise GAG chains. Therefore, in CHO745α2 cells transfection of GFP-syndecan-1 leads to the expression of the unsubstituted core protein. Syndecan-1 core had no effect on CHO745α2 cell adhesion (III, Fig. 6A), suggesting that the HS GAGs would play a key role in the collaboration of syndecan-1 with α2β1 integrin. The significance of syndecan-1 HS GAGs on adhesion to collagen was further studied in MDA-MB-231 cells with similar outcome. Expression of syndecan-1 core protein lacking all three HS attachment sites had no effect on adhesion to collagen while syndecan-1 expression again induced adhesion (III, Fig. 6C), verifying the substantial role of HS GAGs for syndecan-1 support on α2β1 integrin-mediated adhesion.

5.3.3. Syndecan-1 regulates actin organisation and cell signalling on collagen

To explore whether syndecans and α2β1 integrin localise in the same subcellular compartments, immunofluorescence studies were performed. GFP-syndecan-1 or non-tagged syndecan-1 expressed in MDA-MB-231 cells colocalised with α2β1 integrin and F-actin in the protruding membrane ruffles in cells spreading on monomeric collagen (III, Fig. 7A, B). In comparison, GFP-syndecan-2 or -4 or non-tagged syndecan-4 did not localise to membrane ruffles or showed no colocalisation with α2β1 integrin (III, Fig. 7A, B). Furthermore, syndecan-1 silencing caused the disruption of actin organisation in MDA-MB-231 cells spreading on collagen (III, Fig. 7C), phenotype in line with CHOwtα2 cells treated with heparitinase, an enzyme that cleaves HS GAGs (III, Fig. 2B).

Finally, the role of syndecan-1 in α2β1 integrin downstream signalling was investigated. Since α2β1 integrin has been shown to trigger MMP-1 transcription (Riikonen *et al.*, 1995; Langholz *et al.*, 1995), the luciferase reporter gene was placed under the control of the full-length MMP-1 promoter and its activity was studied in CHOwtα2 and CHO745α2 cells. In CHOwtα2 cells, collagen induced a time-dependent activation of the MMP-1 promoter detected as luciferase activity, whereas FN had no effect (III, Fig. 8A). In contrast, in CHO745α2 cells devoid of GAGs, the MMP-1 promoter activity was not induced regardless of the matrix (III, Fig. 8A). Collectively, α2β1 integrin signalling on collagen seems to require HS GAG chains. CHO745α2 cells indeed show slightly higher MMP-1 activity on the GFOGER peptide than on collagen (III, Fig. 8B). However, this was not significant. Eventually, syndecan-1 silencing in MDA-MB-231 cells reduced (III, Fig. 8C) and overexpression of GFP-syndecan-1 induced the activity of MMP-1 (III, Fig. 8D). In summary, these data demonstrate that the supportive role of syndecan-1 in α2β1 integrin-mediated adhesion to collagen also concerns integrin downstream signalling.

5.4. SYNDECANS AND INTEGRINS TOGETHER REGULATE MATRIX REMODELLING AND INVASION IN BREAST CANCER (IV)

5.4.1. α2β1 integrin-mediated collagen contraction and RhoA signalling require GAGs

Integrin α2β1 is the major receptor for type I collagen, especially in 3D collagen matrix (White et al., 2004). We show that unlike on FN, lack of glycosaminoglycans (GAGs) renders α2β1 expressing cells unable to bind collagen and assemble stress fibres, and that syndecan-1 supports α2β1-mediated cell adhesion (III). To study cell-collagen interactions in more *in vivo* like conditions, 3D collagen lattices are used (Eckes et al., 2006). In this setting, cells remodel the matrix by contracting the free floating lattice in an α2β1-dependent manner (Langholz et al., 1995). First, we examined matrix remodelling of MDA-MB-231 cells stably transfected to express the control GFP vector. Cells were seeded within a free-floating 3D pepsin-extracted collagen matrix. GFP-expressing MDA-MB-231 cells embedded in a floating collagen gel were capable of contracting the matrix, whereas α2 function-blocking antibody brutally impaired collagen contraction (IV, Fig. 2A). Signalling through pathways activating RhoA GTPase is involved in collagen matrix contraction by fibroblasts (Rhee and Grinnell, 2006). Using a rhotekin-RBD pull-down assay we show, that RhoA activation in 3D collagen matrix depends on α2 integrin (IV, Fig. 2A). Also, cell invasion into 3D pepsin-extracted collagen requires α2β1 integrin, as α2 integrin function-blocking antibody completely inhibits invasion (IV, Fig. 5A).

To determine whether cell surface GAGs participate in the regulation of cell interaction with 3D collagen, we studied CHO cells. Stable cell lines expressing α2β1 integrin, wild-type CHOwtα2 and the mutant CHO745α2 cells, were embedded in collagen

lattices. CHOwtα2 contracted the matrix efficiently, whereas contraction by CHO745α2 cells was severely impaired (IV, Fig. 2B). Neither cell line was able to contract collagen in the absence of α2 integrin (not shown). These results indicate that cell surface GAGs function in concert with α2β1 integrin in collagen gel contraction.

5.4.2. Syndecan-1 and -4 play supportive roles in collagen matrix contraction and RhoA signalling

We demonstrate that syndecan-1 functions as α2β1 integrin co-receptor in cell adhesion to 2D collagen (III). Here we show, that 3D collagen contraction and 3D collagen-induced RhoA activation are dependent on α2β1 integrin and cell surface GAGs (IV, Fig. 2A, B). Therefore, we wanted to investigate the role of different syndecan proteoglycans in remodelling collagen matrix, utilising the MDA-MB-231 cells (described in III) that express either GFP or GFP-syndecan-1, -2, or -4 with upregulation of syndecan 1.9-fold, 1.4-fold, or 1.5-fold, respectively (III, Fig. S2B, C). GFP-syndecan-1 had only a minor effect on collagen matrix contraction, whereas GFP-syndecan-2 and even more efficiently GFP-syndecan-4 increased contraction by 30±7% and 45±5%, respectively (IV, Fig. 2C; syndecan-2 not shown). These results could not be explained by differencies in proliferation as GFP-syndecans did not have effect on cell proliferation (IV, Fig. S2B). Again, these findings were reproduced in cells transiently overexpressing non-tagged syndecans, even though due to relatively low transfection efficiency (12-22%; III, Fig. S2D) the effects were smaller (IV, Fig. 2D). The ability of syndecans to induce matrix contraction was not dependent on the syndecan expression levels alone. Even though in the stable MDA-MD-231 GFP or GFP-syndecan cells the expression of syndecan-1 is the highest, syndecan-4 induces contraction most efficiently (IV, Fig. 2C). The difference in contraction was reflected in RhoA activity in cells cultured inside collagen for 18 hours. Syndecan-2 and -4 expressing cells displayed higher RhoA activity compared to GFP expressing cells (IV, Fig. 2E; syndecan-2 not shown). Importantly, increased collagen contraction by syndecans remained dependent on α2β1, since α2 function-blocking antibody completely inhibited contraction and reduced RhoA activation in GFP-syndecan-4 cells (IV, Fig. 2E) equally efficiently as in GFP cells inside collagen (IV, Fig. 2A). These data indicate that distinct syndecans cooperate with α2β1 integrin in cell adhesion to 2D collagen and in contraction of 3D matrices. RhoA evokes contractility through its effector ROCK (Chrzanowska-Wodnicka, et al., 1996; Kimura et al., 1996). Therefore, we studied the implication of ROCK in syndecan-promoted collagen matrix contraction. Selective ROCK inhibition using a chemical agent Y27632 evidently impaired contraction in all MDA-MB-231 GFP or GFP-syndecan expressing cells (IV, Fig. 4A) but not to a complete extent as was the consequence of α2 integrin blockade (IV, Fig. 2A, E). ROCK is known to directly increase the phosphorylation of MLC (Totsukawa et al., 2000). In order to understand the effect of integrin-syndecan cooperation on downstream signalling, we investigated whether adhesion to 3D fibrillar collagen could activate MLC.

Indeed, after 3 h on fibrillar collagen, higher MLC phosphorylation was detected in syndecan expressing cells compared to control GFP expressing cells in 6-7 immunofluorescence images quantitated (IV, Fig. 3B).

GFP-syndecan-1 and non-tagged syndecan-1 colocalise with α2β1 integrin and F-actin in the protruding membrane ruffles in MDA-MB-231 cells spreading on 2D monomeric collagen (III, Fig. 7A, B). Conversely, GFP-syndecan-2, GFP-syndecan-4, and non-tagged syndecan-4 are shown not to colocalise in membrane ruffles with α2 integrin (III, Fig. 7A, B). On the other hand, we show here the cooperation between α2β1 integrin and syndecan-4 in collagen matrix contraction. Hence, we investigated whether integrin α2β1 localises differentially with respect to syndecans on a thin layer of pepsin-extracted 3D collagen gel. All of the cells spread efficiently on collagen, forming obvious actin and integrin containing ruffles. However, unlike on 2D collagen (III, Fig. 7A, B), on 3D collagen all three syndecan-1, -2, and -4 colocalise with α2β1 integrin and actin in the protruding ruffles in MDA-MB-231 transfected with non-tagged syndecans (IV, Fig. 3C, scored pixel intensities: syndecan-2 not shown), demonstrating that the same spatial localisation of syndecans may play a role in their ability to cooperate with α2β1 integrin in 3D collagen.

5.4.3. Syndecans prevent breast cancer cell invasion into collagen

Rho GTPases regulate actin dynamics and cell motility. Likewise, syndecans and integrins participate in the regulation of cell migration and interact with MMPs. During invasion, migrating cells degrade the surrounding matrix by means of proteases. To glide along the narrow tunnels generated, propulsive actomyosin contraction forces and spatially regulated integrin adhesion sites are required (Ridley *et al.*, 2003). Ultimately, we investigated the role of syndecans in breast cancer cell invasion into collagen matrix. In MDA-MB-231 cells, expression of syndecan-1 or -4 significantly impaired invasion into the subjacent, pepsin-extracted collagen matrix compared to control GFP expressing cells (IV, Fig. 4A). Again, in parallel with collagen contraction, invasion into collagen matrix remained dependent on α2 integrin since the α2 integrin function-blocking antibody prevented invasion completely (IV, Fig. 1D, 4A). Also, ROCK inhibitor efficiently attenuated invasion in all cells. Consistent with the contraction result, ROCK inhibitor impaired invasion of control cells by 51%. ROCK inhibitor could further diminish the syndecan expression-impaired invasion significantly only in the syndecan-4 expressing cells (IV, Fig. 4A).

5.4.4. Oncogenic Ras signature is behind the interplay between α2β1 integrin, syndecan-1 and -4, and MT1-MMP

Several gene expression signatures reflecting the activation state of an oncogenic pathway have been identified. The genes constituting the Ras oncogenic signature

include *ITGA2* (α2 integrin), *MMP14* (MT1-MMP), *SDC1* (syndecan-1), and *SDC4* (syndecan-4) (Bild et al., 2006). The MDA-MB-231 cell line carries an activating mutation of *KRAS* (G13D) (Kozma *et al.*, 1987). Likewise, we could reproduce the impact of *KRAS* regulation on these genes in MDA-MB-231 cells. Silencing of *KRAS* with an siRNA resulted in donwregulation of *ITGA2*, *MMP14*, *SDC1*, and *SDC4* expression (IV, Fig. 1A). Ras transformation is a conventional process behind EMT, while cells acquire invasive properties and lose cell-cell contacts. To directly determine the role of *KRAS* in MDA-MB-231 cell invasive phenotype, *KRAS* siRNA silenced cells were allowed to invade into pepsin-extracted collagen matrix, resulting in significant, 55% reduction of invasion in the *KRAS* silenced cells compared to the control siScr transfected cells (IV, Fig. 1B). Matrices prepared from pepsin-extracted collagen cannot form intermolecular crosslinks. Fibroblasts or tumour cells invade regardless of MMPs, without generating tunnels (Sabeh et al., 2004). In contrast, membrane-anchored MT1-MMP pericellular collagenolytic activity drives tumour cell invasion through crosslinked, acid-extracted collagen matrix (Sabeh et al., 2004). Given the results of syndecans intensifying α2β1 integrin-mediated invasion, we investigated the role of *KRAS* signature in physiologically relevant, crosslinked collagen invasion. Consistent with the results on pepsin-extracted collagen, *KRAS* silenced cells invaded the crosslinked, acid-extracted collagen matrix 42% less compared to the siScr cells (IV, Fig. 1C).

Further, we explored cell invasion in the absence of different components of the *KRAS* signature. MDA-MB-231 cells were transfected with syndecan siRNAs or MT1-, MT2- and MT3-MMP siRNA or treated with inhibitor for MMPs or α2 integrin function-blocking antibody and finally allowed to invade into 3D crosslinked collagen matrix. Levels of syndecan downregulation acquired by siRNA transfection were moderate (IV, Fig. S3), yet the effects on invasion were rather dramatic. Compared to scrambled siRNA transfected cells, neither α2 integrin function-blocking antibody, the siRNA silencing of MT1-, MT2-, and MT3-MMP, nor the common MMP inhibitor GM6001 allowed invasion at all, but entirely trapped the cells on top of the matrix (IV, Fig. 5A, B). In comparison, silencing of syndecan-1 or -4 induced invasion (IV, Fig. 5A, B). Based on qRT-PCR, syndecan-1 silencing resulted in 2.0-fold upregulation of MT1-MMP expression (IV, Fig. 6A) compared to the scrambled siScr or syndecan-4 siRNA transfected cells. Thus, the augment in invasion after silencing of syndecan-1 was likely due to the higher level of MT1-MMP. Syndecans participate in actin dynamics regulation. Also, they have an influence on cell morphology in 3D collagen (IV, Fig. 5C). Syndecan-1 silencing produce single-cell invasive phenotype: cells have lost cell-cell contacts, broken off from their neighbors, and show several protrusive structures (Fig. 5A, C). Syndecan-4-silenced cells appear in collectives, tightly in contact with their neighbour illustrating prominent cell-cell contacts (IV, Fig. 5A, C). In EMT during development and pathological processes, syndecans are spatially and temporally regulated. Syndecan-4 upregulation has been associated with EMT (Dobra et al., 2003). The appearance of highlighted cell-cell contacts after silencing of syndecan-4 could be due to interference with gene expression essential for the EMT phenotype of cells.

The role of syndecans in the α2β1 integrin-mediated invasion into crosslinked collagen was further confirmed by syndecan expression. Congruent with the data on invasion of the syndecan silenced cells, expression of either GFP-syndecan-1 or GFP-syndecan-4 both effectively inhibited cell invasion into crosslinked collagen (IV, Fig. 4B). As syndecan-1 silencing induced upregulation of MT1-MMP levels, we checked for the effect of syndecan expression on MT1-MMP. Consistent with the silencing results, syndecan-4 expression had moderate 20% reducing effect on MT1-MMP expression and syndecan-1 expression resulted in 49% reduction in the cellular levels of MT1-MMP (IV, Fig. 6B). Hence, means of syndecan-1 regulation over collagen invasion seems to be through control of MT1-MMP expression. Taken together, these data emphasise the interplay between α2β1 integrin, MT1-MMP, syndecan-1, and syndecan-4 regulating collagen invasion as part of the Ras oncogenic pathway signature.

6. DISCUSSION

6.1. Vimentin, EMT, and cancer

The insolubility of IFs and lack of proper tools has made studying IFs difficult whereas modifying the other cytoskeletal systems has long been straightforward. Actin filaments can easily be disrupted in cells using inhibitors latrunculin A or cytochalasin D. Microtubules are targeted with nocodazole and colchicine, among others whereas no specific inhibitor for IFs exists, making removal of them from cells complicated (Ku *et al.*, 1999). This is probably one of the reasons why the existing literature on the cellular functions and relevance of vimentin is relatively thin. Research has treated vimentin as a boring marker and an inert component of the cytoskeleton only, although it is spatially reorganised and dynamically regulated through post-translational modifications, specifically phosphorylation. Generally its presence has been taken as a contaminant for example in proteomics studies aiming to identify protein-protein interactions. However, novel findings on the functions and protein interactions of vimentin presented by us and others broaden the understanding of vimentin as an important and interesting signalling hub and regulator in cancer.

In this thesis study, the role of a novel PKC isoform, PKCε, in the regulation of cell motility in fibroblasts was clarified. Different PKC isoforms control a majority of the identified phosphorylation sites on cytoskeletal regulators. Inhibition of PKCε in fibroblasts impairs directional cell motility and results in the accumulation of β1 integrin into cytosolic vesicles (Ivaska *et al.*, 2002). Luckily, when identifying the partners in cytosolic β1 integrin vesicles, vimentin was not discarded as a contaminant. Instead, integrin traffic was found to be linked to vimentin phosphorylation by PKCε (Ivaska *et al.*, 2005). The results show, that PKCε controls the transport of endocytosed β1 integrins to the plasma membrane. Subsequently, PKCε regulates directional cell motility. Vimentin was demonstrated to be a master player in the process of integrin endocytosis. In the absence of PKC catalytic activity or properly phosphorylated vimentin filaments, integrins become trapped in intracellular vesicles and the directionality of integrin-mediated cell migration is lost.

The initiative for us to study EMT arose from obscurity around the EMT marker vimentin. For long, it has been clear that vimentin expression frequently correlates with the mestastatic conversion of epithelial cells and mesenchymal characteristics as well as with a migratory phenotype. Our mission was to find out whether vimentin expression actually plays a role in the initiation and progression of EMT or rather comes along as a consequence of mesenchymal changes in the cell. Vimentin expression correlates with adverse pathologic effects in breast cancers of young women (Chen *et al.*, 2008). It is selectively expressed in aggressive breast cancer cell lines (Gilles *et al.*, 2003). The human mammary, non-transformed but benign cell line MCF10A that shows moderate expression of vimentin was chosen for analysis. This cell line has been shown to have many features of basal progenitor cells, suggestive of

a multipotent lineage potential (Neve *et al.*, 2006). The changes in gene expression resulting from silencing of vimentin with an siRNA were studied with Illumina cDNA microarray screen. Encouragingly, several migratory and EMT-related genes were differentially expressed, suggestive of a regulatory role for vimentin. However, had we known then that the focus of the study would eventually become the role of vimentin in Slug and oncogenic H-Ras induced EMT, we would have chosen the Slug and/or H-Ras expressing MCF10A cell line and elucidated whether vimentin silencing can revert the EMT phenotype that is induced by Slug and H-Ras. After thorough investigations, we realised that in addition to the hypothesised spontaneous EMT induction, vimentin was in command of EMT in both Slug- and H-Ras-induced EMT. Thus, the focus of our interest shifted. Eventually, we show that removal of vimentin in EMT-like cells reverts the phenotype towards epithelial. In the microarray screen, 85 genes were found to be significantly upregulated and 35 downregulated. Most importantly, not all of the genes were differentially expressed. We chose 11 most interesting of these for further verification based on their putative links to EMT, migration, invasion, or cancer. In addition to verifying the vimentin-regulated alteration in the expression of these genes by TaqMan qRT-PCR (Axl also on protein levels), expression levels of these genes correlated in a wide array of clinical samples and other cell lines, indicative of general value *in vivo* not restricted to certain type of breast cancer cells. Suggestive role of vimentin in the regulation of EMT suddenly became evident in a large amount of clinical material instead of an siRNA effect *in vitro* in a 2D-grown cell line. Such a wide correlation of our results gives a special value to the findings. Justifiably, we believe and declare that identifying vimentin as a key regulator of migratory genes in EMT is a remarkable milestone in EMT research.

One of the genes we show upregulated in MCF10A cells upon vimentin silencing is Rab25 (also known as Rab11c). It regulates receptor endocytosis and induces cell invasion by $\alpha5\beta1$ integrin delivery to the cell front (Caswell *et al.*, 2007). Rab25 seems to have a dual role in cancer. Its expression is restricted to epithelia, and in normal epithelial cells Rab25 displays a protective role. Rab25 expression is mostly lost during tumourigenesis. Vice versa, we show that silencing of vimentin results in upregulation of *RAB25* along with other changes in gene expression towards a more epithelial phenotype. Snail transcription factor triggers EMT and directly binds to *RAB25* and E-cadherin promoter, repressing their expression (De Craene *et al.*, 2005). However, comparative genomic hybridisation analyses showed amplification of *RAB25* in about half of ovarian and breast cancers (Cheng *et al.*, 2004), linking increased expression of Rab25 with shorter survival and tumour aggressiveness by an unknown mechanism. This is contradictory to other, very recent findings that present Rab25 with tumour suppressor function in clinical samples of triple-negative (estrogen, progesterone, and ErbB2 receptor negative) breast cancer. In addition, re-expression of Rab25 in an *in vivo* mouse model substantially suppressed tumour growth (Cheng *et al.*, 2010). In accordance, low Rab25 expression correlated with substantially shorter survival in patients with colorectal carcinomas (Nam *et al.*, 2010). Loss of *RAB25* was earlier demonstrated in breast cancer cell lines quite the contrary to presence in normal mammary epithelium and cultured primary human mammary epithelial cells.

Specifically, all breast cancer cell lines that contain a mutated Ras had lost Rab25, but Rab25 overexpression had no effect on Ras levels (Cheng *et al.*, 2006). These results show an association between loss of Rab25 and tumourigenesis in breast cancer. In view of the fact that controversial studies exist, Rab25 might play a dual role. These reports give a wider significance to our findings of vimentin as a negative regulator of Rab25 expression and their strong inverse correlation in breast tissue and cell lines. However, the manner for vimentin to induce EMT is likely an outcome summing up the regulation of differential expression of several genes rather than affecting only few pathways.

The human Ras GTPase family includes three proto-oncogene members H-Ras, K-Ras, and N-Ras, which contribute to cell growth and differentiation. Activating mutations of certain amino acids induce the activation of the oncogenic proterties of these Ras proteins leading to a constantly GTP-bound form that exhibits dominant transformation actitivity, commonly contributing to tumourigenesis. *RAS* mutation frequency is the highest among any genes in human cancers. Breast cancer cells that exhibit a point mutation of tumourigenesis-associated *RAS* have lost *RAB25* espression (Cheng *et al.*, 2006). To further clarify the mechanism of vimentin in EMT induction, the oncogenic H-Ras-V12G GTPase and the transcription factors Snail and Slug were chosen for experimental investigations. Both H-Ras and Slug expression in MCF10A cells promoted high vimentin and Axl induction. Furthermore, the EMT phenotype triggered by either H-Ras or and Slug was completely dependent on vimentin expression. The effect of Snail on vimentin and Axl expression, and especially the effect of vimentin silencing on Axl under Snail induction was moderate (unpublished results), thus not of eventual interest for us.

β4 integrin is one of the genes that correlate positively with vimentin expression following vimentin silencing in our microarray screen. Importantly, correlation between β4 integrin and vimentin expression was also observed in cancer cell lines and clinical cancer material. Normal tissue samples exhibited no correlation, suggesting that vimentin regulates β4 integrin expression only in pathological conditions. β4 integrin is frequently upregulated in human carcinomas (Mercurio and Rabinovitz, 2001). It is implicated in oncogenesis dependent on HGF-mediated Met RTK activation (Trusolino *et al.*, 2001), and as a mediator of anchorage-independent cancer cell growth (Zahir *et al.*, 2003). *ErbB2* RTK amplification is common in aggressive breast tumours and correlates with poor prognosis (Berger *et al.*, 1988). β4 integrin amplifies signalling of the activated, oncogenic version of ErbB2 to promote mitogenic and invasive signalling during mammary tumourigenesis. In an *in vivo* mouse model of ErbB2-induced mammary cancer, deletion of β4 integrin signalling domain reduced invasive growth and tumour onset (Guo *et al.*, 2006). Increasing evidence suggest, that the RTKs Met, ErbB2, and EGFR promote invasive signalling through β4 integrin phosphorylation, giving special importance on our results that position vimentin as a regulator of β4 integrin expression.

We show that MCF10A cells display EMT-like morphologic and phenotypic changes at the wound edge similarly to an earlier study (Sarrio *et al.*, 2008) elucidating dynamic regulation of vimentin and spontaneous changes in MCF10A cells in response to low cell density. These changes in both of the studies include upregulation of vimentin and Slug, cadherin switching, and cytoskeleton reorganisation. Already a long time ago, MCF10A cells were shown to undergo EMT-like changes upon wounding: vimentin upregulation in cells at the wound edge, induced cell migration, and actin cytoskeleton reorganisation towards a migratory phenotype (Gilles *et al.*, 1999). Also, we demonstrate that higher vimentin expression correlates with enhanced motility in MDA-MB-231 cells compared to MCF10A cells and in SW13vim+ cells compared to SW13vim– cells. Consistently, vimentin silencing in both MDA-MB-231 and MCF10A cell lines significantly abrogated cell motility. Very recently, vimentin expression was linked to morphological and behavioural characteristics of mesenchymal cells (Mendez *et al.*, 2010). The level of vimentin expression correlated with cell shape, motility, and adhesion structure dynamics. Most cell lines are capable of migrating to some extent without vimentin or Axl. This is well expected since neither vimentin nor Axl knockout mice have severe defects, suggestive of compensatory mechanisms. Cell lines experimented by us (MCF10A, MDA-MB-231, SW13) make no exception, but display some residual migration still after vimentin or Axl silencing. Thus, the focus of the study is the role of vimentin and Axl in the EMT-induced migration instead of declaring their overall supremacy during migration.

Our data feature a novel link between vimentin and Axl as one of the EMT-related genes that vimentin regulates. Hand in hand with vimentin expression and cell motility, we and others (Meric *et al.*, 2002) show high levels of Axl expression in MDA-MB-231 cells and substantially lower expression in MCF10A cells. We conducted several attempts to address the exact molecular mechanism by which vimentin influences Axl expression or functions as a critical signalling node inducing EMT. Based on our results, vimentin regulates Axl on mRNA level influencing its transcription. Possibly, vimentin regulates the function or stability of certain transcription factors that determine the level of Axl and other genes whose expression vimentin is in charge of. The transcription factor EHF (also called Ese-3) was upregulated in vimentin-silenced cells based on the microarray data. We expressed EHF in MCF10A cells but detected no influence on Axl expression (unpublished results). In the future, the experiments could include silencing of vimentin in EMT induced by any of the EMT-inducing transcription factors that are the most obvious candidates for regulation by vimentin. Vimentin could influence the function of these transcription factors for example by affecting their stability in a similar manner as it protects the polarity protein Scribble from proteasomal degradation (Phua *et al.*, 2009). IFs could offer cells a common way to regulate transcriptional activity by dynamically governing the homeostasis and stability of transcription factors. Alternatively, we could express these transcription factors in vimentin-positive and vimentin-negative cells such as the SW13vim+ and SW13vim– or cells immortalised from the vimentin wt and knockout mice, and follow subsequent changes in expression of vimentin, Axl, and other EMT-related proteins. Another attempt performed to pinpoint the exact mechanism of vimentin-dependent

induction of Axl was inhibition of DNA methylation. It has been published that DNA demethylation using 5-aza-2'-deoxycytidine induces methylation of the Axl promoter and upregulates Axl expression in cell lines expressing low levels of Axl but not in highly Axl expressing cells (Mudduluru and Allgayer, 2008). Thus, we treated MCF10A cells with the same demethylation agent, 5-aza-2'-deoxycytidine but were unable to reproduce the published data in these cells (unpublished results). Thus, these investigations were not continued with respect to possible epigenetic effects of vimentin expression. The effects of vimentin silencing on cell migration that we find are Axl-mediated could be due to vimentin silencing-induced alterations on the expression of the other genes vimentin regulates. However, the effects obtained by overexpression of Axl in MDA-MB-231 and SW13 cells suggest a major role for Axl, and the levels of reduction in migration reached by Axl silencing alone were similar to the effects of vimentin silencing, supportive of particularly Axl as a major endpoint of vimentin-dependent regulation of motility.

The significance of EMT in embryogenesis and organ development as well as in several adult pathologies is undisputable. Several examples exist of the signalling pathways essential during developmental processes becoming injurious to health and playing dual roles in the different stages of EMT. For example, the deficiency of a Rac GEF called T-lymphoma invasion and metastasis inducing protein 1 (Tiam1) protects mice from Ras-induced tumourigenesis. Tiam1 knockout mice get less chemically induced skin tumours than wt animals. However, the tumours that do develop in Tiam1 knockouts are more likely to progress to malignancy than those in wt mice (Malliri *et al.*, 2002). Tiam1 and activated Rac support E-cadherin-mediated cell-cell adhesion thereby reducing invasiveness in epithelial cells (Hordijk *et al.*, 1997). As another example of the dual roles of signalling pathways, very recently, the adaptor protein Dab2 was shown to to regulate the responses of TGF-β *in vitro* and *in vivo*. Downregulation of *DAB2* has been reported in a multiplicity of human cancers already earlier. In squamous cell carcinoma, *DAB2* was shown to be epigenetically downregulated. Low *DAB2* levels as well as Dab2 protein levels in tumours correlated with poor patient survival, and Dab2 expression was evidenced to function as a switch changing TGF-β from prevention to progression of cancer. (Hannigan *et al.*, 2010).

Initiation of an EMT is often linked with activated Src kinase and growth factor signalling. EMT-inducers evoke phosphorylation of cadherins and catenins and the changes eventually lead to loosening of cell-cell contacts. As a consequence, transcription factors (LEF1, Snail, Slug, Myc) are activated to trigger the expression of downstream targets. In the second phase of EMT, cells become motile. E-cadherin is downregulated while integrin signalling dominates. The third phase is already metastatic: MMPs degrade matrices and cells invade from tissue to another (Thiery, 2002). Our studies of integrin signalling, Axl expression, and Ras involved in cell motility may be settled in the second and third phases of EMT after the initial, inducing sparkle.

6.2. Axl as a drug target in breast cancer

Axl makes an excellent drug target in many ways. In normal tissue, Axl is moderately expressed whereas in many cancers it is highly upregulated. Knockdown of Axl alone has no dramatic effect on the development of mice. Similarly to EGFR, Axl can be targeted both from the extracellular space with antibodies as well as intracellularly with a small-molecule inhibitor. Several studies support the role of Axl in cancer progression *in vivo*. Axl regulates breast cancer metastasis and patient survival (Gjerdrum *et al.*, 2010). A selective small-molecule inhibitor of Axl, R428, impaired angiogenesis, reduced metastasis, and extended survival in breast cancer mouse models (Holland *et al.*, 2010). In accordance, we show in an *in vivo* mouse experiment that silencing of Axl prevents breast cancer cells from extravasating in lung. These cells seemed trapped inside of the vasculature whereas the control cells had penetrated the vessel walls and invaded lung tissue. In conclusion, these data are encouraging and propose to therapeutically target Axl signalling to prevent breast cancer metastasis.

Withaferin-A is a bioactive compound isolated from an Indian medicinal plant. It exhibits a range of pharmacological properties that include anti-tumour and anti-angiogenesis activities. Withaferin-A has been published to bind vimentin (Bargagna-Mohan *et al.*, 2007). However, it has several other targets as well. Recently, in vimentin-expressing mesenchymal cells, Withaferin-A was shown to induce apoptosis and caspase-dependent vimentin degradation. It also abrogated growth, migration, and invasion both *in vitro* and *in vivo* in mice (Lahat *et al.*, 2010), suggesting vimentin as a novel, druggable target in cancer. The mechanisms of Withaferin-A anti-tumourigenic effects include inhibition of Akt phosphorylation and impairing of NF-κB function that are both downstream signalling mechanisms of Axl. This would be in line with our results of vimentin expression regulating Axl expression, suggesting that the actual Withaferin-A mechanism of action might be attenuation of Axl signaling in response to vimentin downregulation.

6.3. The interplay between integrins and syndecans

Several mechanisms of integrin and syndecan cooperation on FN have been emphasised in detail. However, their collaboration in cell-collagen matrix interactions remained elusive until our research. This study comprehensively demonstrates that syndecan-1 functions as α2β1 co-receptor in cell adhesion to collagen whereas syndecan-2 and -4 do not play a role. In the absence of cell surface GAGs, integrin α2β1 is unable to mediate adhesion to collagen or induce downstream signalling to upregulate MMP-1 expression. Furthermore, disruption of cell surface HS PGs leads to disassembly of stress fibres in cells adhering to collagen. Silencing and overexpression studies identify syndecan-1 as an important co-receptor of α2β1 in cell adhesion to full-length collagen but not the specific integrin recognition sequence GFOGER. We also show, that K-Ras upregulates the expression of α2β1 integrin, MT1-MMP, and syndecan-1 and -4 in MDA-MB-231 cells. Syndecan-4 functions as a co-receptor for

α2β1 in collagen matrix remodelling and RhoA activation inside 3D collagen. Syndecan-1 and -4 negatively regulate cell invasion, however via distinct mechanisms: syndecan-1 but not syndecan-4 regulates the expression of MT1-MMP. Likewise, syndecan-1 silenced cells invade crosslinked collagen matrices in a single-cell invasive manner whereas silencing of syndecan-4 triggers cell invasion as collective strands. In conclusion, complex collaboration of integrins and syndecans is highly relevant to cell-collagen interactions and the specific syndecan involved depends on matrix dimension (2D or 3D).

When we initially submitted these results for publication, GFP-tagged expression constructs aroused serious concerns among syndecan researchers. Doubts include expression and proper localisation of syndecans at the plasma membrane as well as the possible hindrance to the intracellular domain interactions caused by the GFP construct. These are valid points since the intracellular domains of syndecans are short and exhibit essential signalling functions. GFP is large and really could physically interfere with these functions when placed intracellularly close to the syndecan tail. To ensure that the GFP constructs retain the same signalling activity, transient expression of native receptors was used in some of the experiments (adhesion, collagen matrix contraction). Furthermore, immunofluorescent and FACS stainings confirm the plasma membrane localisation of the GFP-syndecans. Equal localisation of native receptors and the GFP-syndecans could have been further verified with syndecans from different species and monoclonal antibodies. The possibility of different syndecan expression levels resulting in the discovered changes in capability of cell adhesion and matrix contraction was ruled out by careful investigation. Levels of green fluorescence in the GFP-syndecan cell lines suggest that the relative expressions were syndecan-4>syndecan-2>syndecan-1. However, it is important to take into account also the different levels of endogenous syndecans expressed by MDA-MB-231 cells. Since the syndecan antibodies have very different affinities and the FACS results cannot be compared between syndecans, RT-PCR was utilised to analyse the overall expression levels of the syndecans. Syndecan-2 is in fact expressed 26% more than syndecan-4 and still the strongest contraction inducer is syndecan-4 (syndecan-2; unpublished results). As further evidence for the specificity rather than amount of syndecan-1 as a positive regulator of cell adhesion to collagen, the most abundantly expressed syndecan, syndecan-2, in MDA-MB-231 cells was silenced using an siRNA and examined in adhesion. Clearly, syndecan-2 was not a positive regulator of α2β1-mediated adhesion to collagen in these cells.

Most of the imaginative inventions on the mechanism of the cooperation between integrins and syndecans were vitiated by the fact that PGs seem to be dispensable in adhesion to the α2 integrin specific, trihelical peptide GFOGER. Integrin α2β1 binds collagen via the inserted I domain structure found in the α2 subunit. Three independent, GFOGER-containing binding sites per collagen molecule have been identified for the α2I domains (Xu *et al.*, 2000). In addition, collagen has been shown to contain a separate binding site for syndecan-1. Based on our results, the α2I domain seems capable of binding to GFOGER sequence independently, but in the case of full

length collagen this site is either partially masked when syndecan-1 site is not occupied or some kind of cooperative clustering of α2β1 integrin and syndecan-1 is needed for adhesion to intact collagen. Therefore, we believe that HS GAGs would not promote adhesion alone. Instead, HS chains need to interact with collagen in the context of syndecan-1-supported adhesion. We show a supportive contribution of syndecan-1 in α2β1 integrin-dependent adhesion to collagen. In accordance, a synergistic crosstalk between syndecan and α2β1 integrin on cell adhesion and spreading in response to laminin-derived peptides was published very recently. These peptides consist of active modules binding to either syndecan or α2β1 integrin. Combination of the peptides accelerates cell attachment, spreading, and FAK phosphorylation compared to the single peptides (Hozumi *et al.*, 2010). On fibronectin, consistent reports illustrating syndecan-integrin collaboration constitute a diverse repertoire. Taken together, proof of direct syndecan-integrin receptor crosstalk does not exist, but clear synergy between the downstream signalling cascades of these receptor families is already well presented.

An active site in syndecan-1 extracellular core protein associates with αvβ3 and αvβ5 integrins that are critical in angiogenesis in vascular endothelial cells. Interfering with this interaction using a peptide inhibitor synstatin inactivates the integrins and blocks angiogenesis *in vitro* and in a mouse model *in vivo* as well as mammary carcinoma formation in nude mice (Beauvais *et al.*, 2009). Divalent cations are indispensable for integrin ligand binding and subsequent integrin activation. Depletion of the cations by using divalent cation-free PBS does not block the integrin activation occuring during antibody engagement-induced clustering of syndecan-1 (Beauvais *et al.*, 2004), indicative of a co-receptor role for syndecan-1 instead of functioning as a ligand. However, cells do not spread dependent on syndecan-1 alone, but require integrin. Syndecans and integrins likely form complexes in clusters at the plasma membrane, for example when syndecan-1 is engaged by multivalent ligands. It brings together signalling molecules, kinases that are associated in their cytoplasmic domains, and allows for possible transphosphorylation and activation of signalling transduction. In this thesis study, activation of integrin utilising Mg2+ or Mn2+ did not further promote adhesion of MDA-MB-231 cells (unpublished results). Similarly, the presence or absence of GAGs had no effect on α2β1 integrin conformation in CHO cells. Thus, the mechanism of syndecan collaboration in adhesion must be other than inducing a conformational change on integrin.

Syndecan-4 is known to bind various growth factors, cytokines, MMPs, and cell adhesion molecules, possibly maintaining epithelial cell phenotype. Loss of syndecan-4 expression results in the release of these molecules in the cell surroundings instead of engagement by syndecan-4. This may in part explain the invasive behaviour of syndecan-4-silenced cells demonstrated by us; vice versa, our studies also demonstrate a crucial role for syndecan-1 and -4 in abrogating cell invasion into 3D collagen matrix. Syndecan-4 knockout mice revealed the fundamental functions of syndecan-4, namely focal adhesion assembly and wound healing (Echtermeyer *et al.*, 2001). We show syndecan-4-mediated support of integrin-dependent matrix contraction.

Intensifying this cooperation could be a therapeutic target in the treatment of conditions related to tissue repair: chronic wounds, scarring, and fibrosis.

6.4. Syndecan contribution in cancer

The role of syndecans in tumourigenesis remains obscure. Syndecan signalling effects seem highly context-dependent and clinical findings on syndecan-1 correlation with tumour progression are quite controversial. In accordance with our findings of syndecan-1 knockdown resulting in invasive behaviour in breast cancer cells, in several human cancers, epithelial syndecan-1 expression is reduced. These cancers include hepatocellular carcinoma (Matsumoto *et al.*, 1997), laryngeal cancer (Pulkkinen *et al.*, 1997), head and neck carcinoma (Inki *et al.*, 1994), and non-small-cell lung cancer (Nackaerts *et al.*, 1997). Likewise, head and neck carcinoma patients with higher syndecan-1 expression have a favourable prognosis (Inki *et al.*, 1994). Conversely, syndecan-1 expression is strongly upregulated in pancreatic cancer (Conejo *et al.*, 2000) and breast cancer, its role is highly variable. High syndecan-1 expression was associated with aggressive breast carcinoma phenotype and poor prognosis (Barbareschi *et al.*, 2003), but then loss of epithelial syndecan-1 was identified with strong prognostic value in breast carcinomas (Loussouarn *et al.*, 2008). Differential roles of syndecan-1 in breast cancer are definitively not clarified. Supportive of our results that introduce syndecan-1 as an inhibiting factor in invasion, membrane-bound syndecan-1 was earlier demonstrated to inhibit invasiveness whereas soluble syndecan-1 switched the phenotype to invasive. The controversy among data collected on syndecan-1 expression in carcinomas may derive from different properties of the membrane-bound and the shed, soluble syndecan-1. Not all of the investigations can distinguish between the different forms of syndecans, and samples interpreted as syndecan-negative may actually have lost their soluble syndecans during sample preparation (Nikolova *et al.*, 2009). The contribution of syndecan-4 in tumourigenesis and invasion is not well studied. Syndecan-4 overexpression is implicated in proliferative renal nephropathy disease (Yung *et al.*, 2001) and liver carcinomas (Roskams *et al.*, 1998), but no consistent correlation with differentiation stages of carcinomas or differential expression pattern in tumours have been published. Compared to normal tissue, human placental carcinomas displayed markedly decreased expression and modified localisation of syndecan-4 (Crescimanno *et al.*, 1999). These studies are in accordance of our investigations that present syndecan-4 expression as a protective mechanism in cancer, preventing cell invasion.

The possible mechanisms for syndecans in regulating integrin-dependent cell invasion include at least intracellular traffic, the Rho GTPase family, and other syndecan interactions. First of all, we show induced RhoA activity and matrix contractility in syndecan-4 overexpressing cells. Syndecan-4 null fibroblasts have poor persistence in migration and display dysregulated Rac1 activity. In migrating cells, RhoA-driven contractility at the cell rear pushes the cell body forward, while focused Rac1 activity in the leading edge is essential for persistent migration and matrix protrusion. In short,

the Rho GTPases play major roles in migration and syndecans participate in the regulation of their activity. Therefore, influencing the activity and localisation of Rho GTPases and their GAP, GEF, and GDI regulators in concert with integrins might be one of the mechanisms of syndecan-mediated regulation of invasion evidenced in our studies. In addition to regulating cytoskeletal dynamics, the Rho GTPases also participate in the regulation of intracellular traffic. Secondly, syndecan cytoplasmic tails interact with cytoskeleton and cytosolic proteins such as PDZ-binding proteins. For example, syndecan-4 V region directly associates with PKCα and β1 integrin-dependently with α-actinin (Greene *et al.*, 2003). Thus, syndecans might regulate cellular functions, such as invasion, by functioning as scaffolds; their cytoplasmic tails are known to bind distinct effectors. Syndecan-integrin interplay most probably involves bringing together and combining their cytoplamic interactions, as the β1 integrin-dependent interaction between syndecan-4 and α-actinin suggests.

The extracellular syndecan interactions might also play a major role in syndecan-dependent regulation of invasion. The syndecan HS and CS GAG chains engage various ECM ligands and keep them away from mischief. Syndecans also function as adhesion receptors and contribute to cell adhesion and spreading in collaboration with other adhesion receptors such as integrins. During cell movement, adhesions at the cell rear are released and new adhesion sites are constantly assembled at the leading edge. Strengthening of adhesion is a means of attenuating migration. MT1-MMP plays a key role in invasion. Our data indicate that syndecan-1 overexpression promotes a marked downregulation in MT1-MMP levels, explaining at least partly the concomitant reduction in invasion. Conversely, silencing of syndecan-1 resulted in a two-fold upregulation in MT1-MMP levels and stimulated invasion. The supportive role of syndecan-1 in adhesion and the regulation of MT1-MMP expression probably both influence cell invasion. In addition to the MT1-MMP properties allowing for tissue penetration, MT1-MMP like a few other MMPs can shed syndecan-1. In the case of MT1-MMP, the cleavage of syndecan-1 stimulates cell migration (Endo *et al.*, 2003) and breast cancer cell proliferation (Su *et al.*, 2008). Rab5 GTPase is another protein with capability of shedding syndecan-1 (Hayashida *et al.*, 2008). These data of MT1-MMP-mediated promotion of migration are in line with ours and shedding offers another possibility to speculate on the mechanism.

Syndecans and integrins both can be transported in intracellular vesicles dependent on the Arf and Rab GTPases involving PDZ proteins, phosphoinositide membrane lipids, syntenin, and possibly, syndecan clustering in lipid rafts. The endocytic pathway is another possible mechanism explaining how syndecans regulate cell invasion. From the endosomal compartments, syndecans may return to the plasma membrane through the Arf6 recycling pathway. Furthermore, the extracellular ligands of syndecan bound by its HS GAGs accompany syndecan along the endocytic pathway and get trapped in the intracellular vesicles in certain conditions (Zimmermann *et al.*, 2005). Following internalisation, the consequent reduction in the cell surface amount and activity of the molecules binding to syndecan HS GAGs is likely to influence cellular functions. In summary, the syndecan recycling pathway may affect processes such as adhesion,

spreading, and invasion. Interfering with the endocytosis-related pathways could give new insights into understanding of the mechanisms behind our data regarding the syndecan support in integrin-mediated processes. To investigate whether the endocytic pathways of the syndecans and integrins collaborate, these receptors could be immunofluorescently stained together with markers of the different endosomal compartments to detect possible colocalisation. Internalisation and recycling of α2β1 integrin in the syndecan-overexpressing cell lines or in syndecan-silenced cells in presence and absence of different kinase or GTPase inhibitors could be compared in a biotinylation assay. Furthermore, the contribution of endocytosis to syndecan-mediated reduction in cell invasion could be explored by specifically targeting the different steps of the endocytic pathway. Internalisation of syndecans and integrins and prevention of their recycling back to the plasma membrane could explain how syndecan expression inhibits cell invasion. Taken together, the significance of syndecan signalling in physiologic and pathologic processes is very intriguing. However, at present the details remain elusive and still require intensive research.

7. SUMMARY AND CONCLUSIONS

In this study, the role of intermediate filament vimentin in regulation of integrin traffic, cell motility, and EMT was investigated. The results demonstrate the dependency of intracellular integrin transport and cell migration on vimentin phosphorylation mediated by PKCε. On inhibition of PKC and vimentin phosphorylation, β1 integrins become trapped in cytosolic vesicles and cells lose directional motility. Also, vimentin was declared with EMT-inducing properties and as a regulator of several migratory genes. Specifically, vimentin was shown to possess a fundamental role in the regulation of Slug- or oncogenic H-Ras-induced EMT. Vimentin has earlier been addressed to correlate with cell motility. This study indicates a novel link between vimentin and receptor tyrosine kinase Axl that is strongly associated with cancer. Vimentin is found to regulate cell migration in 2D and 3D matrices Axl-dependently. Lung extravasation of breast cancer cells is abrogated in Axl knockdown cells. In conclusion, Axl is illustrated as a key proximal component of vimentin-dependent cancer cell migration during EMT. Axl signalling most probably will offer novel therapeutic targets for cancer drug design in the near future.

This study also elucidates the cooperational contribution of syndecan proteoglycans and integrin adhesion receptors on cell adhesion, organisation of the actin cytoskeleton, matrix remodelling, and invasion. The results point out a supportive role for syndecan-1 in α2β1 integrin-mediated cell adhesion to 2D collagen whereas syndecan-4 is established as an essential regulator of α2β1 integrin-mediated matrix contraction. Finally, cancer cell invasion was investigated. Oncogenic K-Ras was found to regulate the expression of α2β1 integrin, MT1-MMP, syndecan-1 and -4 and the complex interplay between them in cell invasion. These results clarify fundamental patways regulating cancer invasion and metastasis.

The studies presented in this thesis converge on EMT and integrin signalling: intracellular traffic, adhesion, motility, and matrix invasion. Moreover, our data support the speculations on vimentin functioning as a scaffold protein for signalling proteins; similar properties for syndecans have been proposed.

After decades of intensive research, cancer still causes a tremendous amount of suffering worldwide. The whole cancer research field has bustled with the fascinating dream of individual, specific cancer treatment, but even if long steps have already been taken, only few molecularly accurate therapeutics exist and the objective seems far away in the future. Small streams become fierce rivers; basic research is the steady foundation of novel scientific inventions. All of the molecules - integrins, syndecans, vimentin, Axl, MMPs, and Ras - and phenomena investigated in this thesis study are substantially relevant in oncogenesis. The findings presented here reveal novel molecular interactions for drug targeting and shed light on trails possibly of value in unravelling the mysteries of tumourigenesis.

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