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Samer Abdallah

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THÈSE

Pour obtenir le grade de
Docteur

Délivré par :
UNIVERSITE MONTPELLIER

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Chimiques et Biologiques pour la Santé et de l'unité de
recherche Centre de Recherche en Biologie cellulaire de
Montpellier

CNRS-UMR5237

Spécialité : Biologie Santé

Présentée et soutenue publiquement par

Samer ABDALLAH

**REGULATION DU DETACHEMENT ET DE LA
MIGRATION DES CELLULES EPITHELIALES
CANCEREUSES PAR L'INHIBITEUR DE
L'ACTIVATEUR DU PLASMINOGENE DE
TYPE-1 (PAI-1)**

Soutenue le 20/04/2016 devant le jury composé de :

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“SUCCESS IS THE SUM OF SMALL EFFORTS, REPEATED
DAY IN AND DAY OUT.” – ROBERT COLLIER

Dedicated to My Father

لى روح نبي الطاهر

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LIST OF ABBREVIATIONS

NUMBERS

2D, 3D Two-Dimensional, Three-Dimensional
3D-MCTS Three-dimensional Multi cellular tumour spheroids

B

bFGF basic Fibroblast Growth Factor
BSA Bovine Serum Albumin

C

CAAX Cysteine; Aliphatic Amino acid, any amino acid (X)
CAF Cancer-Associated Fibroblast
CAM Cell Adhesion Molecules
CD44 Cluster of Differentiation 44
CDK Cyclin-dependent kinase
CTC Circulating Tumour Cells

D

DNA Deoxyribonucleic acid

E

E-Cadherin Epithelial Cadherin
ECM Extra cellular matrix
EGF Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
EMT Epithelial-Mesenchymal Transition
ERK Extracellular signal-Regulated Kinase

F

FA Focal Adhesion
F-Actin Fibrillar- Actin
FAK Focal Adhesion Kinase
FCS Fetal Calf Serum

G

GAGs Glycosaminoglycans
GF Growth Factor

GPI

GlycosylPhosphatidylInositol

 **H** 

HER2

HGFR or C-Met

HRP

Human Epidermal Growth Factor Receptor 2

Hepatocyte Growth Factor Receptor

HorseRadish Peroxidase

 **I** 

IL-6

InterLeukin-6

 **J** 

JAK

JNK

Janus Kinase

c-Jun NH2-terminal Kinase (MAPK family)

 **L** 

LRP

Low-Density Lipoprotein Receptor-Related Protein

 **M** 

MAPK

MAT

MCTS

MET

MLC

MMPs

MRCK

Mitogen-Activated Protein Kinase

Mesenchymal-Amoeboid Transition

Multi cellular tumour spheroids

Mesenchymal Epithelial Transition

Myosin Light-Chain

Matrix Metalloproteinases

Myotonic dystrophy kinase-related CDC42-binding kinase

mRNA

MT1-MMP

Messenger Ribonucleic acid

Membrane Type-1 Matrix MetalloProteinase

 **N** 

N-CAM

NEDD9

NP-40

N-WASP

Neural Cell Adhesion Molecule

Neural precursor cell Expressed, Developmentally Down-regulated protein 9

Tergitol-type NP-40 (nonyl-phenoxypolyethoxyethanol)

Neural Wiskott-Aldrich syndrome protein

 **P** 

PAI-1

PAK

PAs

Plasminogen Activator Inhibitor type-1

p21 Activated Kinase 1

Plasminogen activators

| | |
|--------------|--|
| PAS | Plasminogen Activator system |
| PBS2- | Phosphate Buffer Salin without calcium and magnesium |
| PI3K | Phosphatidylinositol-3-Kinase |
| P-MLC | Phosphorylated Myosin Light-Chain |
| pRb | Phosphorylated Retinoblastoma protein |

R

| | |
|--------------|--|
| Rac-1 | Ras-related C3 botulinum toxin substrate 1 |
| Ras | Rat Sarcoma |
| Rb | Retinoblastoma protein |
| RhoA | Ras homolog gene family, member A |
| RNA | Ribonucleic acid |
| ROCK | Rho-associated protein kinase |

S

| | |
|-------------------|---|
| SDS | Sodium Dodecyl Sulfate |
| SERPIN | Serine Protease Inhibitor |
| SFK | Src family kinase |
| SH2 domain | Src Homology 2 domain |
| Src | v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog |
| STAT | Signal transducer and activator of transcription |

T

| | |
|-------------------------------|---|
| TGF-β | Transforming Growth Factor- β |
| TIMPs | Tissue Inhibitors of Metalloproteinases |
| t-PA | Tissue-type Plasminogen Activator |

U

| | |
|-------------|--|
| uPA | Urokinase-type Plasminogen Activator |
| uPAR | Urokinase Plasminogen Activator Receptor |

V

| | |
|--------------|---------------------------------------|
| VEGF | Vascular endothelial factor |
| vLDLR | Very Low Density Lipoprotein Receptor |

W

| | |
|-------------|--|
| WASF | Wiskott-Aldrich syndrome protein family |
| WASP | Wiskott-Aldrich syndrome protein |
| WAVE | Wiskott-Aldrich syndrome protein family member |

PREFACE

This thesis is the final work of my PhD research which stems from a collaborative work between the groups headed by G. Barlovatz at the Evry University and this of P. Roux in Montpellier. I started by mid-September 2012 at Evry university in the Paris region, at IBISC laboratory where I conducted my research till the end of December 2012 under the direction of Pr. Georgia Barlovatz-Meimon. Afterwards, I completed my research at Montpellier University, at the National Center for Scientific Research (CNRS) from January 2013 till this day under the direction and the supervision of Dr. Véronique Gire, Pr. Georgia Barlovatz-Meimon and Dr. Pierre Roux. My thesis book presents the results I have obtained so far to characterize the role of Plasminogen Activator Inhibitor Type-1 (PAI-1) in cell detachment and migration of non-invasive epithelial cancer cells.

My thesis starts with an introduction composed of 3 chapters synthesizing a review of the literature about tumour development and cancer, as a first chapter, in which I discuss the hallmarks of cancer occurrence shared by almost all malignancies that enable tumour growth and metastatic escape. In the second chapter, I discuss in details a cancer hallmark (cell migration and invasion) that represents a prerequisite for metastasis. The third chapter discusses the up-to-date knowledge about the involvement of PAI-1 in cancer cell detachment, migration and invasion.

Next, my results are described in an article format that we intend to submit very soon.

I end this work by a discussion in which I compare my results with those previously obtained and described in the literature and propose a model that summarizes my research work.

Finally, I wish you a very pleasant reading of my thesis.

INTRODUCTION

INTRODUCTION

Cancer is a leading cause of death with approximately 8.2 million cancer related deaths; 14 million new cases in 2012 and the annual cancer cases are expected to rise to 22 millions within the two next decades (world cancer report 2014; <http://www.who.int/mediacentre/factsheets/fs297/en/>). Very early diagnosis, more accurate diagnosis and targeted treatments to prevent relapse will lead to more effective cancer treatments and less therapeutic failures, easing the burden on global health system and decreasing the number of cancer deaths worldwide.

Cancer is a genetic disease and develops in a multi-step process. Normal cells respond to intrinsic and extrinsic signals in a manner that ensures cell homeostasis and thus maintenance of normal tissue architecture and function. Cancer cells are defective in regulatory circuits of proliferation and homeostasis and therefore ignore signals that order them to stop growing or enter apoptosis. This progressively drives sustained proliferation and formation of a local tumour. However, the most detrimental aspect of cancer is the cells' ability to escape from the primary tumour and spread to distant location sites in the body and to form metastasis (Chambers, Groom et al. 2002). Around 90% of deaths from solid tumours are caused by metastases, as such it is of vital importance to understand how metastasis occurs and the underlying processes guiding cell motility and invasion (Nguyen and Massague 2007). Cancer types have been categorized by the type of cells or organ from which they originate and the level of malignancy. The majority of life threatening, aggressive cancers occur in epithelial tissues and give rise to carcinomas.

Both genetic predisposition and environmental factors contribute to the process of tumour formation, but the importance of different influences on cancer formation is largely dependent on the particular type of cancer. Most of the known carcinogens are mutagens that generate mutations in DNA, resulting in activation of tumour promoting (oncogenes), or loss of tumour suppressing (suppressor) genes and stability genes. Activation of oncogenes can be caused by mutational events such as chromosomal translocations, gene amplifications, deletions, aneuploidy or point mutations in the gene. Activation of only one allele of an oncogene is generally enough to confer a selective advantage to the cell. For tumour suppressor genes, inactivation of both alleles by deletions, insertions or epigenetic silencing of the gene is usually required (Luo, Solimini et al. 2009). The process of tumour formation is

understood to begin with a somatic mutation within a cell of a particular organ. Over time the originally mutated cell or clone will acquire additional mutations and the cancer will gain sub-clones that may have acquired characteristics different from the original cell that bestow a survival advantage (Kreso, van Galen et al. 2014). This view is strongly supported by early studies where genetic mutations were analysed across different stages of colorectal cancer (Vogelstein, Fearon et al. 1988).

It is only recently with the advancement in technology that there has been an increase in understanding of the way that tumours are formed and how the mutations that cause them arise (Garraway and Lander 2013). DNA sequencing technologies have revealed that single nucleotide variants are the most prevalent mutation found in human tumours, although only a small amount of these actually alter the DNA sequence that codes for a protein, and this change may confer a proliferative and selective advantage (Loeb 2011). DNA sequencing has also shown that the stepwise accumulation of these mutations is the key to cancer formation and can be used to trace the evolution and clonal expansion of a tumour, allowing for a greater understanding of the complexities of tumour progression (Forment, Kaidi et al. 2012), (Greaves and Maley 2012).

CHAPTER 1. TUMOUR DEVELOPMENT AND CANCER

Cancer development is a long process and many changes need to occur in a normal cell in order for it to develop into a fully transformed malignant cell. Many barriers have to be breached during the course of tumour progression, but cancer cells use different strategies to progress toward overt malignancy. This is evident from the fact that a high degree of heterogeneity in mutations has now been identified in different cells within the same tumour and in the same tumour type derived from different individuals (Lawrence, Stojanov et al. 2013), (Alexandrov, Nik-Zainal et al. 2013), (Kandoth, McLellan et al. 2013). Disruption of many different signalling pathways may lead to the same phenotype in different cancer cells. On the other hand, deregulation of a specific pathway in one cell type may not result in the same phenotypic changes in another cell type (Hanahan and Weinberg 2000), (Massague 2004). In addition, the chronological order of events leading to malignancy also varies between cancer types, which makes it difficult to predict the multi-step process of tumourigenesis. Nevertheless, cancer cells acquire a number of hallmarks shared among almost all malignancies that enable tumour growth and metastatic dissemination (**Figure 1**).

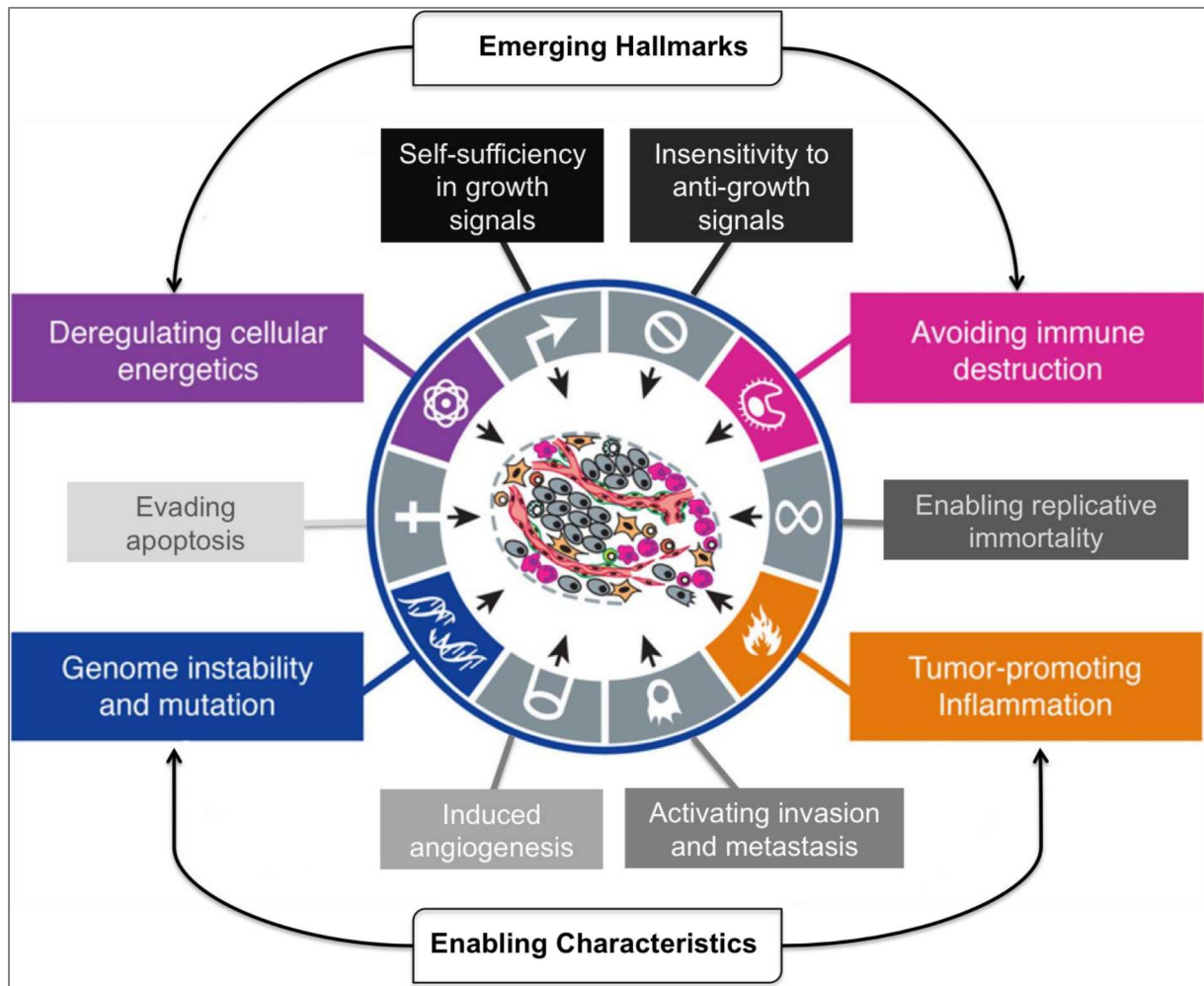


Figure 1. Emerging hallmarks and enabling characteristics of cancer.

An increasing body of research suggests that two additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Because neither capability is yet generalized and fully validated, they are labelled as emerging hallmarks. Additionally, two consequential characteristics of neoplasia facilitate acquisition of both core and emerging hallmarks. Genomic instability and thus mutability endow cancer cells with genetic alterations that drive tumour progression. Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in their inadvertent support of multiple hallmark capabilities, thereby manifesting the now widely appreciated tumour-promoting consequences of inflammatory responses. Hallmarks in degraded grey colours are the six main hallmarks described in Weinberg Cell 2000.

Adapted from (Hanahan and Weinberg 2000), (Hanahan and Weinberg 2011).

The six main hallmarks are self-sufficiency in growth and proliferation signals, insensitivity to anti-growth signals, resisting apoptosis, the ability to proliferate indefinitely (immortalization), genome instability, induction of angiogenesis, and invasive and metastatic abilities (Hanahan and Weinberg 2000), (Hanahan and Weinberg 2011), (Kroemer and Pouyssegur 2008), (Luo, Solimini et al. 2009). There are also two emerging hallmarks of cancer: reprogramming energy metabolism and evading immune destruction. These changes in the cells are acquired during the evolution of tumour and enable progression and genomic instability. Some of these properties are considered as a prerequisite for transformation, while others are associated with malignancy and could contribute to cancer progression in several ways. For the purposes of my thesis project, the hallmark of note is invasive and metastatic abilities and will be presented in more detail in section 2.

1-1 Self-sufficiency in growth and proliferation signals

Extracellular growth signals transmitted through trans-membrane receptors are required for normal cells to divide and proliferate. Anti-growth signals are likewise required to regulate growth. In tumour cells, however, deregulation of both of these control mechanisms leads to increased and uncontrolled proliferation. Anti-proliferation signals usually force cells into a reversible quiescent G0 state that is accompanied by permanent differentiation. Normal cells highly rely on exogenous mitogenic stimulation at G1 for proliferation, which is mostly coordinated by the retinoblastoma protein (Rb) pathway (Massague 2004). Hypophosphorylated Rb binds to and inhibits E2F transcription factors from regulating many genes required for DNA replication whose expression is required for cell cycle progression from G1 to S phase (Massague 2004). Phosphorylation of Rb proteins by mitogen-activated, cyclin-dependent kinases (CDKs) cancels Rb-mediated repression. The Rb pathway is disrupted in human cancers for example by overexpression of cyclin D1 and CDK4 as well as the silencing of p16INK4a and pRb itself (Vogelstein and Kinzler 2004), (Polager and Ginsberg 2009). Loss of Rb weakens these controls, dissociating the cell cycle machinery from extracellular signals, dampening the ability of proliferating cells to exit the division cycle and compromising the execution of Rb-dependent differentiation programs in certain tissues.

The tumour suppressor p53 is a transcription factor that is activated in response to stress signals and functions to stop cell cycle progression in normal cells by inducing

expression of the Cdk inhibitor p21Cip1. The majority of carcinomas bear distinct genetic or epigenetic alterations that disrupt both the p53 and Rb pathways, and as a consequence display impaired cell growth control in G1 phase of the cell cycle (Polager and Ginsberg 2009).

Cancer cells find ways to activate cell proliferation in the absence of exogenous mitogenic stimuli, which make them refractory to normal homeostatic regulation (Hanahan and Weinberg 2000). Signalling through receptor tyrosine kinase (RTK) pathways for example often leads to transcription of growth-promoting genes and many genes involved in these pathways are altered in cancer (Takeuchi and Ito 2011). The upstream receptors themselves, such as epidermal growth factor receptor (EGFR), Hepatocyte Growth Factor Receptor (HGFR) or Human Epidermal Growth Factor Receptor 2 (Her2) are often overexpressed in cancers and tumour cells can also make their own mitogens (Yamamoto, Yuyama et al. 2006). One important pathway downstream of many RTKs is the Ras-MAP-kinase pathway and in many human cancers, Ras proteins are structurally modified so that they signal continuously (Bos 1989). The cell adhesion receptors integrins often regulate the same pathways as growth factor (GF) receptors and crosstalk between these two receptor types is used to stimulate proliferation (Ivaska and Heino 2011), (Khwaja, Rodriguez-Viciano et al. 1997), (Miyamoto, Teramoto et al. 1996). For example, the Src family kinase (SFK) Fyn is activated upon integrin Extracellular Matrix (ECM) binding and binds to and phosphorylates the adaptor protein Shc. This creates a binding site for the Grb2/Sos complex that activates the Ras-MAP kinase pathway (Wary, Mariotti et al. 1998). Grb2/Sos can also be recruited to focal adhesions (FAs) via binding to focal adhesion kinase (FAK) (Schlaepfer, Hanks et al. 1994). Integrin-mediated adhesion to the ECM is usually required for normal cells to progress through the cell cycle. Expression levels of cyclin D1, phosphorylation of Rb and activity of cyclin E-Cdk2 is adhesion-dependent (Zhu, Ohtsubo et al. 1996). Also, the c-Jun NH2-terminal kinase (JNK) regulates cyclin D1 and progression through G1; JNK activation requires integrin ligation and the association of FAK with Src and p130CAS (Ip and Davis 1998). The actin cytoskeleton, whose regulation is largely integrin dependent, also plays a role in cell cycle control. Integrin ligation and activation of the actin regulators Cdc42 and Rac1 has been shown to induce proteasomal degradation of the Cdk inhibitor p21Cip1 in different cell types (Bao, Thullberg et al. 2002).

Phosphatidylinositol 3-kinases (PI3Ks) are a family of proteins activated through

many different signalling agents. PI3Ks phosphorylate phosphatidyl inositol phosphate lipids to generate phosphatidylinositol 3,4,5 trisphosphate (PIP3), which in turn creates docking sites for proteins that carry domains able to bind to the phosphorylated inositol head group. A major signalling pathway downstream of PI3K is mediated via Akt kinase whose activation ultimately leads to increased proliferation and cell growth by inactivating GSK-3 β , reducing the levels of p21Cip1 and p27Kip1 and activating mTOR (Vivanco and Sawyers 2002). The PI3K pathway is deregulated in many types of cancer, often because of decreased activity of the PIP3 phosphatase PTEN, which is frequently mutated or lost in human cancers. Activating mutations in the catalytic subunit p110 α of PI3K are also common in solid tumours (Lee, Park et al. 2005), (Samuels and Velculescu 2004). The Akt pathway is also closely involved in regulating energy metabolism. Cancer cells undergo a bioenergetic reprogramming that bestows properties necessary for successful tumour formation and metastasis. Increased glucose uptake and lactate production indicates high utilization of the glycolytic pathway. Glycolysis normally takes place when oxygen levels are low, but cancer cells increase glycolysis even in conditions of high oxygen. Although glycolysis alone produces less energy than using oxidative phosphorylation in mitochondria, it is thought that acidification of the microenvironment that follows lactate production promotes tumour invasion and that glycolytic intermediates are more beneficial for the cancer cell as they can be shunted into various anabolic pathways (DeBerardinis, Mancuso et al. 2007), (Hatzivassiliou, Zhao et al. 2005), (Martinez-Zaguilan, Seftor et al. 1996), (Vander Heiden, Cantley et al. 2009). Lactate by stimulating the production of hyaluronan and the expression of its membrane receptor, CD44, enhance cell motility and ECM looseness (Stern, Shuster et al. 2002).

Metabolic reprogramming thus provides cancer cell a growth advantage through increased biosynthesis of amino acids, nucleotides and lipids. These alterations could reveal therapeutic targets, as cancer cells become dependent on alternative metabolic pathways for their survival due to their increased energy needs.

1-2 Cellular immortalization

Normal human cells have an intrinsic mechanism that limits their proliferative capacity to a defined number of divisions. A cancer cell has to ensure replicative potential in order to develop into a macroscopic tumour. A cell that can proliferate indefinitely is

immortal and most tumour cells lines growing in culture seem to have this ability, which suggest that immortality is a prerequisite for cell transformation. The ability to proliferate limitless is the main mean to fix the successive genomic alterations that drive clonal expansion and progression in cancer (Artandi and DePinho 2010). The loss of ability to divide of normal cells maintained in culture is known as replicative senescence and is triggered by telomere erosion (Hayflick and Moorhead 1961), (Bodnar, Ouellette et al. 1998). Telomeres are nucleoprotein structures that cap the ends of chromosomes and prevent the natural end of chromosome from being recognized as a double-stranded DNA break. This function protects the chromosomes from enzymatic degradation, inappropriate recombination and end-to-end fusion (Griffith, Comeau et al. 1999), (de Lange 2005). The majority of human cells do not express sufficient levels of telomerase to counteract the end-replication losses that occur as a consequence of semi-conservative replication of linear DNA molecules (Olovnikov 1971). Progressive telomere erosion ultimately results in the loss of telomere function and a permanent proliferative senescence arrest. Senescence can be overcome in human fibroblasts in the absence of the tumour suppressor proteins p53 and Rb (Shay, Pereira-Smith et al. 1991). Allowing cells to proliferate beyond senescence is, however, not enough for immortalization of human cells. Inactivation of checkpoint response allows cell division to continue until cells eventually encounter another proliferative barrier, known as cellular crisis, due to extreme telomere dysfunction. This ultimately leads to chromosomal fusions causing concomitant genomic rearrangement, massive cell death and crisis (Maser and DePinho 2002). Many cancer cells show defects in senescence-controlling signalling pathways downstream of telomere erosion (i.e. p53 and Rb pathways). Cancer cells have found a way to enable unlimited replication and telomere length maintenance by reactivation of telomerase, which adds telomere repeats de novo at the chromosomal ends (Greider and Blackburn 1985).

The majority of human cells do not express sufficient levels of telomerase and it is though that crisis, while leading to the death of most cells in a population, would produce a rare immortal cell which has acquired telomerase activity and extensive cancer-associated genomic changes (Artandi, Chang et al. 2000), (Counter, Hilton et al. 2002). Thus, telomere shortening may both restrict and facilitate neoplastic transformation (Hanahan 2000), (Maser and DePinho 2002).

1-3 Insensitivity to anti-growth signals

In their course to malignancy, transformed cells must not only get provision of their own mitogenic signals but also overcome certain homeostatic anti-proliferative stimuli such as those triggered by an excess cell number or those that are concomitant with the process of cell differentiation (Hanahan and Weinberg 2000). Noteworthy, such anti-proliferative signals are thought to be regulated in many cases at the membrane receptor level (Hanahan and Weinberg 2000), as normal cells usually establish *in vivo* numerous protein-protein interactions with the surrounding cells (i.e. through cadherins and (Cell Adhesion Molecules CAM's)) and with the ECM (i.e. through integrins) in order to evaluate if they are located in the right niche as well as whether cell division is required to compensate for cell loss. For instance, one of these cell-cell interactions, which is mediated by a yet poorly identified membrane receptor (Gradl, Faust et al. 1995), regulates the contact-inhibition response (Dietrich, Wallenfang et al. 1997), (Faust, Dolado et al. 2005), which induces a rapid cell cycle exit when high cellular densities are achieved. Both the p16Ink4a and p27Kip1 cell cycle inhibitors seem to be key regulators of the contact inhibition response (Polyak, Kato et al. 1994), (Wieser, Faust et al. 1999). Consequently, the usual disruption of these pathways in cancer may explain, at least in part, why virtually all cancer cells are unresponsive to contact inhibition and have the potential to grow without temporal (see 1-2) and spatial restrictions.

Another powerful source of anti-proliferative signals is the process of cellular differentiation, in which pluripotent tissue stem/progenitor cells give rise to all the specialized cell types of a multicellular organism. Cell differentiation is indeed a progressive process in which cells steadily lose their proliferative capacity, to finally exit the cell cycle in an irreversible fashion at the stage of terminal differentiation. Of note, the vast majority of somatic cells in our organisms are either terminally differentiated or on the way to this non-replicative State. In contrast, it is a well-known feature of cancer that human tumours show differentiation defects (Potter 1978), meaning that virtually all human tumour cells are de-differentiated to some degree. However, it has been controversial for many years whether the undifferentiated nature of cancer arises from a differentiation back-tracking of already differentiated cells or from a block in differentiation of stem/progenitor cells (Gisselsson 2007). Although no hypothesis has been discarded yet, the latter seems to be favoured (Bapat 2007), (Krivtsov, Twomey et al. 2006), (Widschwendter, Fiegl et al. 2007), to the extent that a new paradigm in cancer is arising and thus pointing to cancer as a stem/progenitor cell

disease (Wicha, Liu et al. 2006). Accordingly, the ability of several oncogenes to block cell differentiation has been proposed to mediate their transforming activity, for example Notch in breast, pancreatic and lymphoid cancers (Sjolund, Manetopoulos et al. 2005) and Bcr-Abl in leukaemia (Klein, Feldhahn et al. 2006).

1-4 Evading apoptosis

Apoptosis or programmed cell death regulates the lifespan of normal cells and eliminate cells after contact with certain toxic insults or following their detachment from the ECM. Apoptosis is initiated by either the signalling of death receptor or the release of cytochrome c from the mitochondria, which in turn, activates a cascade of intracellular proteases of the caspase family (Letai 2008). A significant number of proteins involved in the regulation of apoptosis have been found silenced or deregulated in most cellular and animal models for cancer, as well as in human tumours (Johnstone 2002). The well-known molecular alterations that are important for apoptosis resistance include reduced function of pro-apoptotic molecules, increased expression of anti-apoptotic proteins and increased activity of pro-survival signalling molecules (Igney and Krammer 2002). Defective apoptosis response is required in cooperation with increased proliferation signalling to mediate initial oncogenesis and tumour progression. Furthermore, insensitivity to apoptosis allows tumour cells to escape from elimination by cytotoxic immune cells and anti-cancer treatments. At the molecular level, several mechanisms have been proposed to underlie the acquisition of such malignant phenotype, including the mutation or silencing of pro-apoptotic proteins (i.e. Fas, p53, Bax, Apaf-1) (Johnstone 2002), the overexpression/activation of pro-survival factors (Akt, Ras, Bcl-2, BclxL), enhanced DNA-repair activity (Kohno, Uchiumi et al. 2005) and the alteration of the protein composition of the plasma membrane (i.e. integrins) (Reddig and Juliano 2005).

Anoikis is a specific form of a caspase-mediated apoptosis induced upon loss of cell adhesion or inappropriate cell anchorage (Frisch and Francis 1994). The induction of anoikis, program occurs through the interplay of two apoptotic pathways, either the intrinsic pathway due to the perturbation of mitochondria or the extrinsic pathway triggered by cell surface receptors (Gilmore 2005), (Frisch and Screaton 2001). Anoikis prevents a detached cell from reattaching to an inadequate or inappropriate matrix and can be viewed as a physiological process, which ensures proper development and tissue homeostasis. Cells are protected from anoikis while adherent on appropriate matrix. Binding of integrin proteins to the ECM provides pro-survival signals, which helps in establishing appropriate cell-matrix interactions.

Any association of detached cells with inappropriate matrix would lead to activation of proapoptotic genes owing to incorrect integrin engagement, and consequently anoikis (Frisch and Ruoslahti 1997), (Giancotti and Ruoslahti 1999). Specific integrins have different abilities to protect cells from apoptosis and anoikis suggesting that they utilize diverse signalling pathways. The integrin engagement by ECM leading to anoikis protection triggers activation of FAK, Src kinase, integrin-linked kinase (ILK), converging on master regulators of anoikis resistance, PI3K/Akt or protein kinase B (PKB), and extracellular signal-regulated kinase (ERK). These pro-survival routes promote on one hand the expression and/or activation of anti-apoptotic proteins (Bcl-2, Bcl-X, NF- κ b) and on the other hand, the inhibition of pro-apoptotic members (Bad, Bim), thereby preventing the intrinsic pathways of cell death (Reddig and Juliano 2005), (Paoli, Giannoni et al. 2013). Integrin engagement also suppresses the expression of Fas receptors, thus interfering with the activation of the extrinsic machinery. GF receptors, activated both in ligand dependent or independent manner, synergize with integrins to modulate cell survival (Sharma, Bell et al. 2007). Intercellular adhesion mediated by cadherins or other cell surfaces molecules, activates signalling pathway similar to those triggered by ECM-adhesion (Kantak and Kramer 1998), (Haller, Kunzendorf et al. 1997).

Cancer cells are resistant to anoikis, which is a prerequisite for anchorage-independent growth and survival of a cancer cell in foreign microenvironments. Anoikis resistance emerged as a hallmark of malignant tumours allowing cancer cells to survive in the blood stream and migrate in the absence of proper ECM contacts to colonize distant organs (Frisch and Sreaton 2001), (Chiarugi and Giannoni 2008), (Taddei, Giannoni et al. 2012). Cancer cells adopt different mechanisms to resist anoikis (**Figure 2**). Many cancer cells switch their integrins expression, in order to express the appropriate integrins thereby adapting to a different microenvironment. Undergoing epithelial-mesenchymal transition (EMT) is an essential process for the acquisition of anoikis resistance, too. During EMT, several genes, such as Snail, Twist, HGF-R/cMet and NF- κ B, are upregulated and play a critical role for evading anoikis by constitutively activating pro-survival signal (Thompson, Newgreen et al. 2005). Another strategy adopted by cancer cells to evade anoikis is through constitutive activation of pro-survival pathways such as activation of SFK, the PI3K/Akt cascade, and ERK pathways (Bouchard, Demers et al. 2007), (Davies, Koul et al. 1999), (Paoli, Giannoni et al. 2013). Another plausible protection from anoikis implicates the increase in reactive

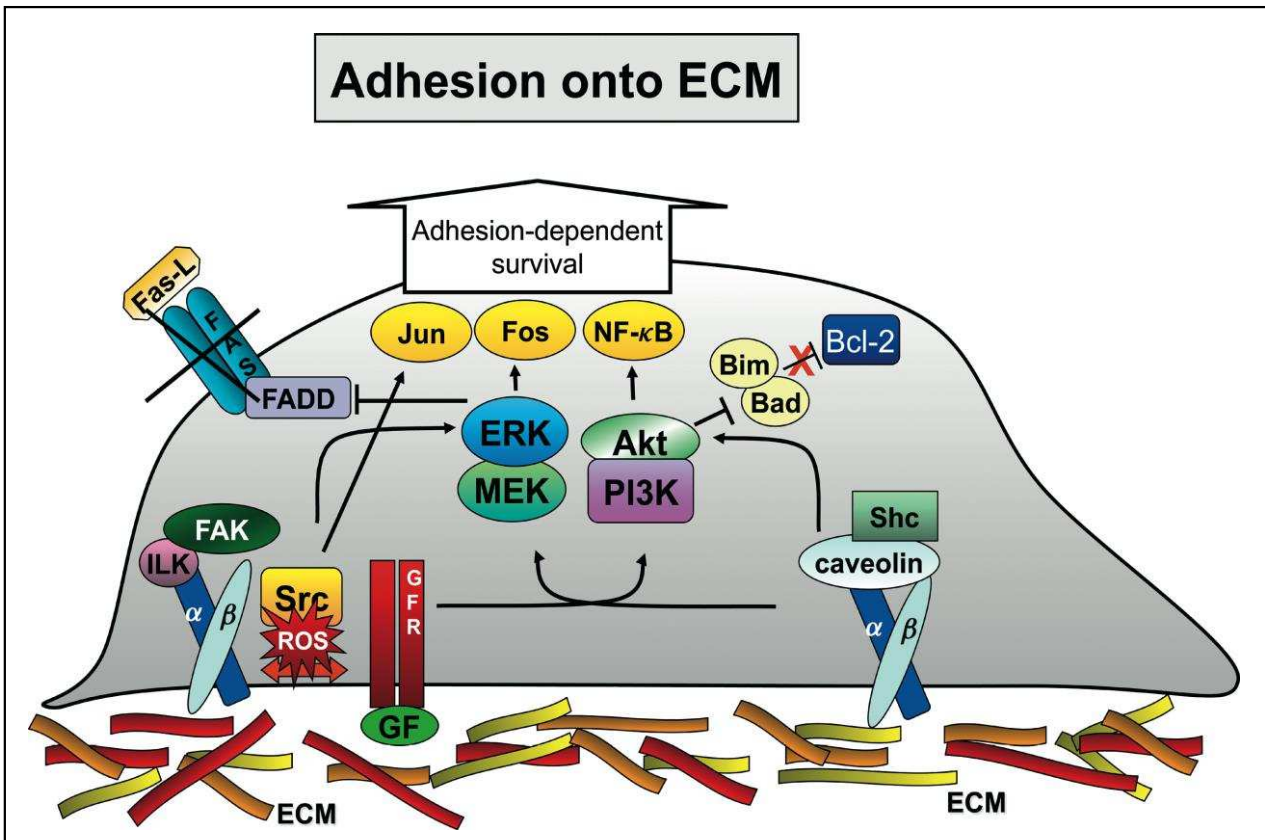


Figure 2. Anoikis resistance promotes metastatic dissemination of cancer cells.

In healthy conditions, anoikis is activated after cells have lost ECM contact. In pathological situations (such as cancer), cells can avoid anoikis. Due to several strategies, cancer cells acquire anoikis resistance, allowing them to survive in the blood stream and colonize distant tissues, where metastatic colonies will grow.

Taken from (Taddei, Giannoni et al. 2012).

oxygen species (ROS, byproducts of metabolic processes) in tumour cells.

The hypoxic environment prevailing within a tumour also helps in promoting anoikis resistance by upregulating different survival and proliferation genes (Whelan, Caldwell et al. 2010). Overall, these mechanisms enable cancer cells to adapt to stress condition by overcoming anoikis. Of note, the ability of cancer cells to elude anoikis induced by growth conditions *in vitro* has been long recognized as the best predictor for their potential tumourigenicity *in vivo* (Shin, Freedman et al. 1975).

1-5 Sustained angiogenesis

It is usually assumed that a cell has become tumourigenic once it has successfully acquired all the cancer-associated traits described above (Hanahan and Weinberg 2000). This assumption is indeed usually validated in “soft-agar” experiments by the cells ability to form microscopic tumour-like spheroids *in vitro* (Shin, Freedman et al. 1975), (Dolado, Swat et al. 2007). However, it has been observed experimentally that many cells that are “tumourigenic” *in vitro* or that have been derived from human tumours or metastatic nodules are unable to produce macroscopic tumours in immuno-depleted mice (Naumov, Akslen et al. 2006). This apparent contradiction is now starting to be understood. Indeed, whereas the above mentioned neoplastic properties are able to drive a cell-autonomous transformed state *in vitro*, further cell expansion *in vivo* into macroscopic tumours additionally requires a process called angiogenesis (Folkman, Merler et al. 1971), (Folkman 1990). In this, tumour cells of the incipient microscopic tumour stimulate the vascular system to provide them with new blood vessels, which then constitute a sustained supply of oxygen and nutrients to the tumour, as well as a way-out for its catabolites, and consequently promote tumour growth. The key relevance of angiogenesis for macroscopic tumour growth is such that it has been proposed that targeting tumour angiogenesis might be a way to turn cancer into a chronic and asymptomatic disease (Naumov, Akslen et al. 2006), (Pincock 2005). In the absence of sufficient vascularisation most tumours cannot exceed a few mm³ in volume and remain clinically silent (Naumov, Akslen et al. 2006). Angiogenesis is a multi-step process involving different cell types, in particular endothelial, perivascular cells (i.e. pericytes, smooth muscle cells), inflammatory and stromal cells. Tumour angiogenesis is initiated at discrete time points during tumour progression (“angiogenic switch”) (**Figure 3**), which are determined by the balance between the genetic status of the tumour itself, signals from stromal and recruited inflammatory cells and by the appearance of hypoxia (Bergers and Benjamin 2003).

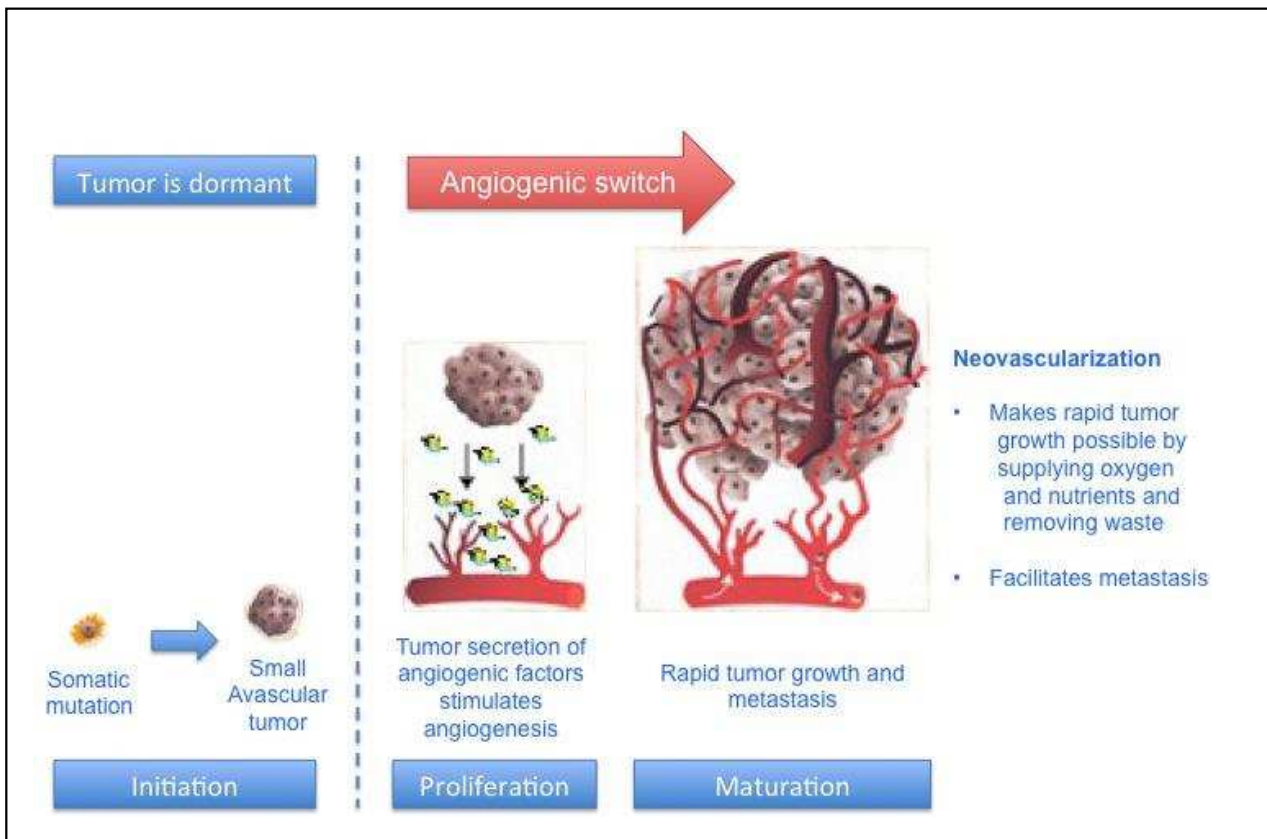


Figure 3. The classical angiogenic switch.

The angiogenic switch is a discrete step in tumour development that can occur at different stages in the tumour-progression pathway, depending on the nature of the tumour and its microenvironment. Most tumours start growing as avascular nodules (dormant) until they reach a steady-state level of proliferating and apoptosing cells. The initiation of angiogenesis, or the 'angiogenic switch', has to occur to ensure exponential tumour growth. The switch begins when tumour mass secrete angiogenic factors that leads to angiogenic sprouting, new vessel formation and maturation, and the recruitment of perivascular cells. Blood-vessel formation will continue as long as the tumour grows, and the blood vessels specifically feed hypoxic and necrotic areas of the tumour to provide it with essential nutrients and oxygen.

Taken From (Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000 ; Bergers G, Benjamin LE. Tumourigenesis and the angiogenic switch. Nat Rev Cancer. 2003).

Tumour angiogenesis goes through two phases separated by the “angiogenic switch”: an avascular phase and a vascular phase (Bergers and Benjamin 2003). In the avascular phase, the dormant tumour is in a steady State of proliferation and apoptosis (Bergers and Benjamin 2003). Only a small subset of the avascular tumours reaches the vascular phase in which exponential tumour growth occurs due to tumour angiogenesis (Bergers and Benjamin 2003). Molecular mediators and regulators of angiogenesis include diffusible factors, such as vascular endothelial factor (VEGF) and basic Fibroblast Growth Factor (bFGF) that can signal quiescent vasculature to begin angiogenesis (Folkman 1995). Negative regulators of angiogenesis, such as thrombospondin-1 and endostatin, must also be decreased for tumour angiogenesis to occur (Folkman 1995). Different factors such as hypoxia in the tumour can cause an increase in VEGF secretion from tumour cells and surrounding stromal cells (Bergers and Benjamin 2003). The tumour suppressor gene p53 upregulates thrombospondin-1, p53 is lost in half of all cancers, which could lead to a decrease of thrombospondin-1 (Hanahan and Weinberg 2011), (Bergers and Benjamin 2003).

The blood vessels formed through tumour angiogenesis differ from blood vessels formed through normal angiogenesis. Vessels formed by tumour angiogenesis do not become quiescent, and the constant growth of new vessels within a tumour causes them to have a different physiology (Bergers and Benjamin 2003). Structurally, tumour blood vessels are dilated, irregularly shaped, have dead ends, and can have cancers cells integrated into the vessel wall (Bergers and Benjamin 2003). The blood vessels are not organized into normal structures like capillaries, arterioles, and venules: they are instead chaotically organized with excessive vessel branching (Baluk, Hashizume et al. 2005). The blood flow in tumour vessels is erratic, it is slow moving and sometimes oscillating; this leads to acidic and hypoxic regions in tumours (Bergers and Benjamin 2003). Tumour vessels are leaky due to overproduction of VEGF and thus experience microhemorrhaging (Bergers and Benjamin 2003). There is high interstitial pressure within the tumour due to the leakiness of the tumour vasculature (Baluk, Hashizume et al. 2005).

Tumour vessels have altered endothelial cell-pericyte interactions, which results in defective surrounding basement membrane (Baluk, Hashizume et al. 2005). The endothelial cells within the tumour vessel have abnormal gene expression and require GFs for survival (Baluk, Hashizume et al. 2005). Overall, tumour vessels are functionally and structurally different than normal blood vessels.

CHAPTER 2. CELL MIGRATION AND INVASION DURING METASTASIS

The acquisition of metastatic behaviour is the optimal achievement of the cancer cell and underlies the malignant nature of cancer. In fact, the presence of metastases at cancer diagnosis easily impedes efficient cancer treatment, with chemotherapy constituting a mere palliative in such cases (McVie 1999), (Vogelstein and Kinzler 2004). The spread of the tumour cells to secondary sites of the body is a complex process involving reciprocal interactions between cancer cells and their stroma (Joyce and Pollard 2009). The classical view on the metastatic cascade includes 6 independent steps (Vogelstein and Kinzler 2004): (1) detachment from the primary tumour mass, (2) the invasion into nearby tissue through degradation of and migration through the microenvironment and the basement membrane layer, (3) intravasation into the blood/lymphatic vessels, (4) passage into the circulation to other “fertile” body sites, (5) extravasation, and (6) colonization and final re-growth at distant site of the body (Gupta and Massague 2006) (**Figure 4**). Each step creates some obstacles to the spread of malignant cells and cancer cells have to overcome all the those obstacles to successfully metastasize (Chambers, Groom et al. 2002), (Geiger and Peeper 2009). Only a small fraction of cells in the primary tumour are able to undergo all the necessary changes in order for metastasis to occur.

Several alterations have been suggested to drive the metastatic phenotype at the molecular level (Hanahan and Weinberg 2000), although it is not clear yet whether these normally result from the acquisition of a specific “metastatic” gene expression program (Gentile and Comoglio 2004), (Minn, Gupta et al. 2005) or are just a consequence of the multiple mutations acquired through the cell transformation process (Nowell 2002). Such alterations include misexpression of cell-cell adhesion proteins such as cadherins (i.e. Epithelial-Cadherin (E-cadherin)) and other cohesive CAMs (i.e. Neural Cell Adhesion Molecule (N-CAM)), which is observed in most invasive tumour cells and is likely to facilitate their detachment from the primary tumour (Hanahan and Weinberg 2000). In addition, not only the tumour cells of malignant tumours, but also the surrounding stromal (Comoglio and Trusolino 2005), (Tlsty and Hein 2001) and inflammatory cells (Baniyash 2006), (Karin, Lawrence et al. 2006), (Philip, Rowley et al. 2004), are known to produce extracellular proteases (i.e. Matrix Metalloproteinases (MMPs) and urokinase-type plasminogen activator (uPA)) that degrade the ECM and create a path to facilitate the invasion of the metastatic cells.

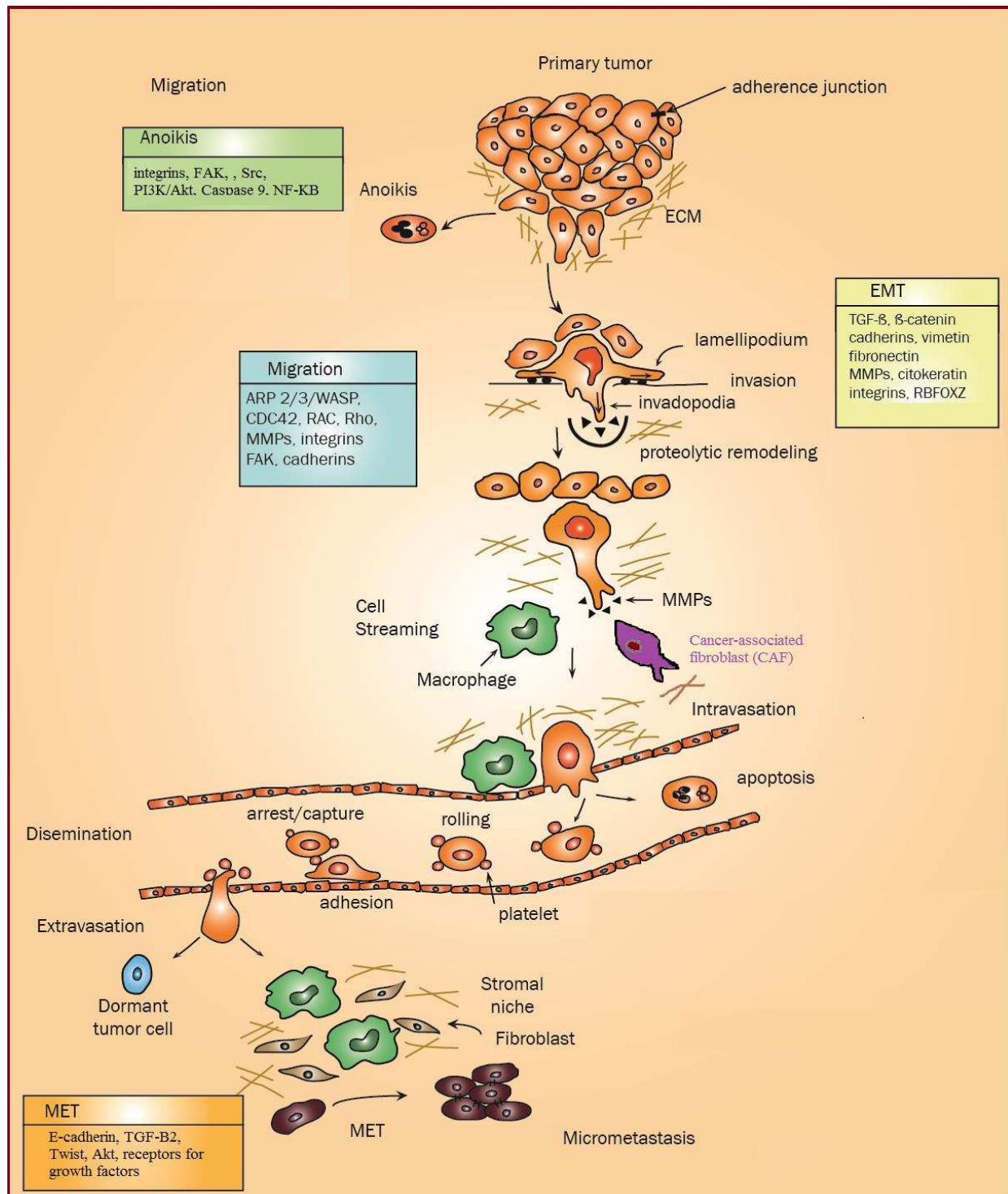


Figure 4. Metastatic cascade.

Separation of tumoural cells from a primary tumour and its migration to reach a blood or lymphatic vessel for dissemination to a secondary site is a very complex process that includes interactions with stromal components and changes in the expression of multiple genes, such as genes involved in cell adhesion, survival, chemoattraction, GFs. Some examples of those genes are shown in the figure.

Adapted from Hernandez-Caballero et al, INTECH open access Publisher 2013.

Importantly, metastatic cancer cells usually change their expression pattern of integrins from those that bind the intact epithelium ECM proteins to others that show higher affinity for degraded stromal components (Hanahan and Weinberg 2000), which probably directs their migration through the degraded epithelium towards the blood vessels. Interestingly, tumour cells do not metastasize stochastically, meaning that specific primary tumour types metastasize preferentially to certain distant organs, for example mammary tumours to bone, liver and lung, stomach tumours to ovary or lung tumours to brain (Patanaphan, Salazar et al. 1988). This reproducible phenomenon was first described in 1882 by Fuchs and colleagues and later proposed as the “seed and soil” theory in cancer (Paget 1889). This means that primary tumour cells (the seed) from a certain tissue of origin are predisposed to colonize and expand only in a particular “fertile” secondary tissues (the soil), a process that we know these days to be largely influenced by the establishment of the suitable biological interactions (i.e. chemokines/chemokine receptors) between the host tissue and the metastasizing cancer cells (Valastyan and Weinberg 2011), (Muller, Homey et al. 2001), (Muller, Sonkoly et al. 2006).

2-1 Tumour cell dissemination and EMT

2-1-1-Composition of the ECM

ECM is present within all tissues and organs of the body and provides strength and elasticity to organs and protects them by a buffering action that maintains water retention and extracellular homeostasis. The physical, topological and biochemical composition of the ECM is tissue-specific. The ECM provides architectural structure supports, functions as a barrier and an anchorage site, provides tracks for the movement of cells, supports signalling by binding and presenting GFs to their respective receptors and possesses biomechanical properties that regulates cell behaviour (Lu, Takai et al. 2011). The ECM is composed of macromolecules, locally secreted by resident cells. The two main classes of macromolecules are polysaccharide Glycosaminoglycans (GAGs) chains, usually covalently linked to specific proteins in the form of proteoglycans, and fibrous proteins of two functional types, structural (collagen, elastin) and adhesive (fibronectin, laminin, Vitronectin, etc.). GAGs are negatively charged heteropolysaccharides composed of repeating disaccharide units and differences in the type of monosaccharide in the repeating unit and their sulfation result in chondroitin sulfate, heparan sulfate, dermatan sulfate and keratan sulfate GAGs (Afratis, Gialeli et al.

2012). Hyaluronan is a non-sulfated GAGs that lacks a core protein. Proteoglycans such as perlecan, lumican, aggrecan, decorin and hyaluronan form a hydrophilic gel-like substance and enable the matrices to withstand high compressive (Jarvelainen, Sainio et al. 2009). Based upon their direct involvement in cell-cell and cell-ECM interactions, these molecules are implicated in the regulation of cell movement. Fibrillar collagens are the most abundant proteins in the interstitial matrix, and provide tensile strength, regulate cell adhesion, facilitate chemotaxis and cell migration (Lu, Weaver et al. 2012). A specialized ECM, basement membranes separate endothelia and epithelia layer from the underlying connective tissue. Most basement membranes are composed of type IV collagen, laminin, the glycoprotein nidogen and the perlecan (Yurchenco 2011). The composition and the structure of basement membranes can likely be modified in many ways to create specialized or context-specific assemblies (Uechi, Sun et al. 2014). Basement membranes, far from being a static support structure are in fact, through directing alterations in cellular behaviour, an active regulator of cell polarization and shape tissue morphology. Changes to basement membranes deposition, structure and composition might organize tissue architecture (Morrissey and Sherwood 2015). Another group of ECM molecules called matricellular proteins including thrombospondin 1 and 2, secreted protein acidic and rich in cysteine (SPARC) also known as osteonectin, tenascin-C, Plasminogen Activator Inhibitor Type-1 (PAI-1) and osteopontin do not function as structural elements but modulate cell-matrix interactions and cell functions (Jarvelainen, Sainio et al. 2009), (Murphy-Ullrich and Sage 2014). The original members of the matricellular family, thrombospondin 1, tenascin-C and SPARC were primarily de-adhesive and pro-migratory molecules (Sage and Bornstein 1991).

The ECM is constantly being remodelled to suit the needs of the tissues and matrix remodelling is especially important in developmental processes such as branching morphogenesis, angiogenesis and in maintaining stem cell niches in different organs. Cells likewise respond to differences in biomechanical properties of the matrix and the interaction between cells and the ECM is therefore highly dynamic. Defects in the processes regulating ECM dynamics can consequently be devastating for tissue homeostasis and contribute to cancer (discussed below).

2-1-2 Cell-matrix interactions

Cell adhesion to the substrate surface elicits integrin mediated FA formation to connect the ECM with intracellular signalling and cytoskeletal complexes. Integrin clustering and causative stress fiber and cellular extension formation enables cellular forces to exert upon the substrate, facilitating cell migration. Force-generating cytoskeletal components transmit internal tensile forces, known as the contractile traction force, to the ECM via FAs. Cell-substrate adhesions occur at both the forward and rearward ends of the cell to facilitate forward migration through a substrate. FAs formed at the cell front persist as cell-ECM anchors until the cell body has migrated over the FAs such that the FAs reach the cell rear.

2-1-2-1 Focal adhesions

FA generates traction forces and it organizes transmission of signals from the ECM to the cell to stimulate pathways involved in cell survival, proliferation and migration. FAs are formed through the recruitment of a multitude of signalling and structural proteins to activated integrin tails at the interface of the plasma membrane and the ECM. FAs are dynamic structures located in lamellipodia regions with a capacity for rapid turnover. More than 150 proteins have been identified as adhesion-associated (Zaidel-Bar and Geiger 2010). The protein composition of adhesions fluctuates even within a single adhesion and is dependent on, for example, adhesion age, ECM composition and substrate tension, among others (Wolfenson, Lavelin et al. 2013), (Zaidel-Bar, Ballestrem et al. 2003).

Cell motility requires an optimal balance of cell adhesion, too little, the cell cannot generate enough traction to move, too much, and the cell is unable to translocate. During polarized cell migration FAs act to stabilise cell adhesion to the ECM to allow the contraction of actomyosin, which enables the cell body to translocate. FAs are constantly assembled and turned over at the leading edge and disassembled at the rear of the cell mediating rear-end detachment (Nagano, Hoshino et al. 2012). Early stages of FAs assembly is a Rac dependent process characterised by the sequential recruitment of specific proteins to the integrins such as, talin, paxillin, vinculin, α -actinin, FAK and vasodilator-stimulated phosphoprotein (VASP) (Zaidel-Bar, Ballestrem et al. 2003, Zaidel-Bar, Cohen et al. 2004) (Zaidel-Bar et al., 2003; Zaidel-Bar et al., 2004; (Zamir, Katz et al. 1999). At this point, the FA may either disassemble or elongate and grow. This is characterised by a RhoA activation switch from

Rac signalling and local mechanical force generated by actomyosin contractility. Spatial location of the FAs relative to the trailing and leading edges is thought to be a trigger of FAs maturation and to some extent disassembly (Berginski, Vitriol et al. 2011) (**Figure 5**). How spatial localisation and mechanotransduction integrate with the signalling mechanisms regulating the dissolution of FAs requires further investigation. It is currently unknown what the minimum signalling and FAs composition requirements are for cell migration.

As the cell protrudes its membrane through the growth of actin filaments, the cell body needs to move forward. This is achieved through actin retrograde flow, which occurs because of the opposing tension in the plasma membrane to the polymerisation of the actin during cell membrane protrusion. Actin retrograde flow pushes the actin network back to the cell body. Lamellipodia adhere to the substrate by the formation of FAs and together with actomyosin contraction and the forces of the retrograde actin flow, the traction required for forward propulsion and thus translocation of the cell body is achieved (Sixt 2012). The linkage between the actin cytoskeleton and FAs is often described as a molecular clutch (Vicente-Manzanares, Choi et al. 2009).

2-1-2-2 Integrins

Cell growth relies on local secretion of ECM molecules to adhere to the surrounding substrate, exhibiting anchorage dependency. The necessity for cell tethering to a surface indicates the ability of cells to sense their environment, which occurs through integrins, a family of transmembrane cell surface adhesion receptors. Integrins are alpha and Beta heterodimers formed by selective pairing between 18 alpha and 8 beta subunits. Integrins contained a large extracellular domain that binds the ECM and links the actin cytoskeleton through a short cytoplasmic tail (Hynes 2002). There are 24 distinct integrin receptors, and their binding specificity is determined by extracellular domain, that recognizes various matrix ligands (Luo, Carman et al. 2007). Integrins bind to specific motifs within the matrix proteins and changes in the integrin repertoire can correlate with changes in migration mode and invasive phenotypes.

As integrins lack intrinsic catalytic activity, they are activated upon engagement with the ECM. Integrins provide a bi-directional conduit for mechanochemical information across the cell membrane, providing a link between the ECM and the interior of the cell.

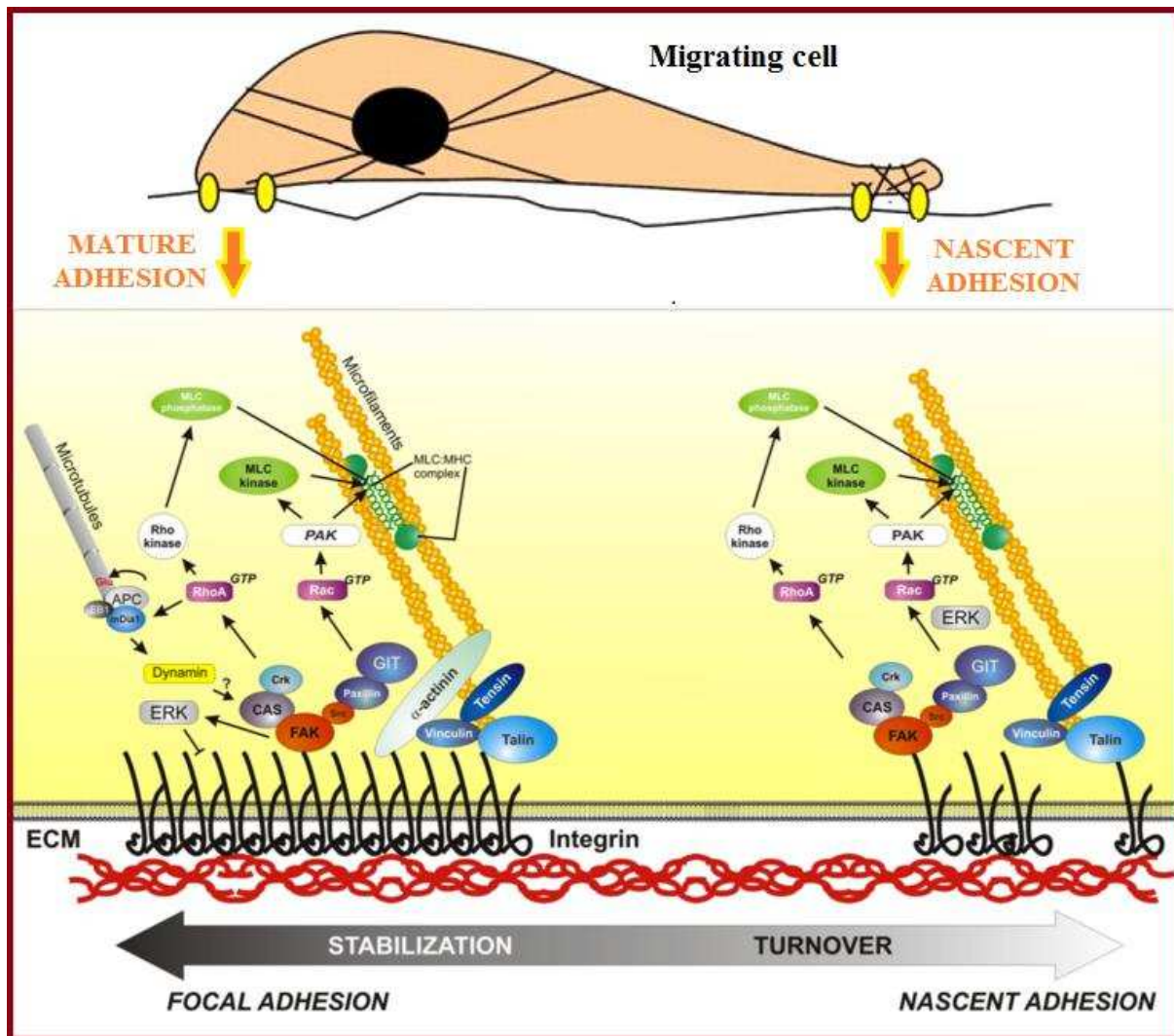


Figure 5. Scheme depicts the process of adhesion maturation.

Nascent adhesions (on the right) are connected to the actin cytoskeleton by an actin linkage that includes talin, vinculin and α -actinin; other signalling adaptors are also recruited to these complexes, including FAK and paxillin. These adhesions generate signals that activate Rac, promoting actin polymerization and preventing myosin II engagement in the lamellipodium. These signals are also required for the dissolution of some adhesions (turnover) as the cell advances. Some adhesions mature into FAs; this process is characterized by an enlargement and elongation of the complexes as well as the appearance of new players, like tensin. Rho activation is thought to generate FAs and actin bundling due to increased myosin II activity. Other events that occur at adhesions are internalization of the integrins, which is mediated by dynamin; and microtubule targeting, which may contribute to adhesion disassembly.

Taken from (Vicente-Manzanares, Webb et al. 2005)

, <https://www.cellmigration.org/topics/adhesion.shtml> and (Wu, Mao et al. 2012).

Cell adhesion to the ECM transmits information via integrin receptors that regulates intracellular signalling via outside-in signalling, which is important, for example, in cell spreading and cell migration. Conversely, intracellular signals can induce changes in integrin conformation and activation that prolong its ligand-binding activity in a process termed inside-out signalling. Integrin engagement with matrix can also affect integrin activation, providing bi-directional crosstalk between inside-out and outside-in signalling (Ginsberg, Partridge et al. 2005), (Luo, Carman et al. 2007).

Integrin-containing adhesion functions as signalling centers orchestrating a network of signalling pathways that mediate cell migration. The Rho GTPases act as a convergence point for these networks and function to regulate actin polymerization and dynamics as well as adhesion itself (Ridley, Schwartz et al. 2003), (Huttenlocher and Horwitz 2011) (discussed below). Signalling by adhesions can be highly localised and thereby drive the polarized phenomena that comprise migration. In addition, the adhesions in protrusions particularly near the leading edge are thought to regulate actin polymerization and thereby localize protrusion (Galbraith, Yamada et al. 2007), (Choi, Vicente-Manzanares et al. 2008). In this way, the transient localized activation of intracellular signalling regulated by integrins contributes to temporal and spatial activation that mediates polarized cell migration.

2-1-2-3 The JAK/STAT3 Pathway

Integrin induced signalling pathways that transmit external cell signals to modulate gene expression and regulation of FAK signalling are also transduced by other downstream pathways, such as the JAK/STAT pathway. This pathway is dynamic in its ability to target genes and regulate key proteins that are primarily involved in orchestrating cell movement and invasion. Of the seven STAT family members (STAT1, 2, 3, 4, 5A, 5B and 6) (Aaronson and Horvath 2002), STAT3 has been consistently implicated in promoting cell migration and invasion (Avalle, Pensa et al. 2012), (Devarajan and Huang 2009) as well as metastasis (Gu, Dagvadorj et al. 2010) in a large number of cancer cell types.

For efficient cell migration and proliferation, target genes within a cell must be activated for transcription. The JAK/STAT pathway has been established as a key signal transduction pathway that transmits stimuli from extracellular moieties and transmembrane

receptors to the cell nucleus in order to regulate gene expression. Binding of GFs or cytokines to their specific cell-surface receptors, results in the activation of members of the Janus kinase family (JAK), a nonreceptor tyrosine kinase, is localized to the cytoplasmic face of the receptor where it creates docking sites for phosphotyrosine protein binding. The phosphorylated (activated) form of JAK proteins subsequently phosphorylates any localized signal transducers and activators of transcription (STATs) at a single tyrosine residue around amino acid 700 (i.e. Y705 in STAT3) within the SH2 domains of STAT, causing STAT transcription factors to dimerize, translocate to the nucleus where it governs the expression of target genes involved in proliferation, survival, invasion and angiogenesis (Darnell 2014), (Stark and Darnell 2012). Mitogen-Activated Protein Kinases (MAP) kinases (ERK, JNK, and p38MAPK) regulate STAT as well by mediating a second phosphorylation event on a serine residue (Ser727 in STAT3) resulting in a fully activated transcription factor (Decker and Kovarik 2000). Known upregulated genes include Fas, Cyclin E1, HGF, VEGF, Transforming Growth Factor-Beta (TGF- β), hypoxia-inducible factor1- α (HIF1- α), Akt, c-Myc, c-Jun, c-Fos, matrix metalloproteinase 2 and the anti-apoptotic genes Bcl-xL, Mcl1 and surviving (Yu, Pardoll et al. 2009). Thus, activation of STAT3 has a broad impact on the expression of genes that promote tumour initiation and progression.

Hyperactive STAT3 is present in most cancers. Conversely, in normal cells the JAK/STAT signalling cascade is spatially and temporally regulated by cellular inhibitors, namely the suppressors of cytokine signalling (SOCS) and tyrosine phosphatases, which interfere with JAK binding to membrane-associated receptors or with STAT phosphorylation to effectively terminate signal transduction initiated by GFs or cytokines (Yu and Jove 2004). Within the context of the JAK-STAT pathway, SOCS3 has been shown to bind Tyrosine-phosphorylated JAK1 and target it for degradation by the proteasome (Boyle, Zhang et al. 2009), (Ungureanu, Saharinen et al. 2002). The SOCS-1 and SOCS-3 genes are also potential regulators of FAK signalling as they regulate tyrosine phosphorylation of FAK and also promote its proteasome-mediated degradation (Liu, Cote et al. 2003). Furthermore, hypermethylation of CpG islands within the SOCS3 promoter, which blocks SOCS3 expression, is detected in hepatocellular tumours and can drive tumour cell migration through enhanced STAT3 and FAK1 activation (Niwa, Kanda et al. 2005).

2-1-3 Epithelial Mesenchymal Transition

The ability of cancer cells to leave the tumour mass depends on losing cell-cell contact, which has been associated with a change in cell shape referred to as EMT. EMT is a complex manifestation of epithelial plasticity (Thiery 2002). Epithelial cells are polarized, adhere and communicate with each other through specialized intercellular junctions and are positioned on a basement membrane *via* their basal surface. EMT, is one of the initiating steps of primary tumour invasion during which a polarized epithelial cell undergoes morphological changes resulting in a motile mesenchymal phenotype (Lamouille, Xu et al. 2014). EMT is characterized by loss of epithelial characteristics, such as apical-basal polarity, E-cadherin and cytokeratin, and activation of mesenchymal genes expression, such as N-cadherin and vimentin. EMT is often accompanied by increases in the matrix metalloproteases secretion and large changes in the production of ECM protein potentially enabling cells to detach from each other (*via* E-cadherin cleavage) and penetrate the basement membrane (Kalluri and Weinberg 2009), (Hu, Kuang et al. 2015). Upon EMT, cells can move away to other sites when environmental conditions of the primary tumour become hostile and detrimental. EMT is a highly regulated process both spatially and temporally during embryogenesis (Thiery, Acloque et al. 2009) and also plays a role in the process of inflammation and wound healing (Thiery 2003). Indeed, EMT programs are encountered in three distinct settings, which are dependent on the physiological context and display different functional consequences (Kalluri and Weinberg 2009). Type 1 EMT occurs in embryo development (as early as gastrulation) and organogenesis (Lim and Thiery 2012), type 2 EMT is associated with wound healing, tissue regeneration and organ fibrosis (Kalluri and Weinberg 2009), (Zeisberg and Kalluri 2013) and type 3 occurs with cancer progression (Kalluri and Weinberg 2009) and is associated with cancer stem cell properties (Scheel and Weinberg 2012). Reactivation of EMT by cancer cells confers enhanced motility and invasiveness during cancer progression thus allowing metastasis. In cancer, features of EMT among cancer cells have been observed in the invasive front of breast (Micalizzi, Farabaugh et al. 2010), prostate (Sethi, Macoska et al. 2010), ovarian (Vergara, Merlot et al. 2010) or colon (Bates, Pursell et al. 2007) cancer clinical specimens. According to the hypothesis that developmental programs are reactivated during tumorigenesis and contribute to tumour progression, many EMT regulators in development are also inappropriately expressed in human cancer and associated with features of EMT, although with less order and coordination than observed in developmental EMT (Gavert and Ben-Ze'ev 2008). However, success in identifying EMT-transitioning cells *in*

in vivo has been hindered by the spatial and temporal heterogeneity of EMT in human tumour and lack of reliable EMT markers that can distinguish tumour cells having undergone EMT from surrounding stromal cells (Voulgari and Pintzas 2009). One explanation for this is the dynamics and transient nature of EMT in physiological context. The inverse mesenchymal to epithelial transition (MET) also occurs and may account for the reversal of cells that have undergone EMT back to the epithelial phenotype. Similarly, at later stage of metastasis, MET would be required for establishment of metastatic colonies in the target organs (Polyak and Weinberg 2009). Presence of EMT markers associated with cells that have undergone EMT should not necessarily be observed in metastasis. The fact that most EMT-inducing changes are transient in nature and do not involve permanent genetic alterations in EMT-regulators support the notion that the phenotypic switching would be important for successful metastasis rather than the EMT *per se*. In this regard, a high degree of epithelial plasticity probably better reflects the *in vivo* situations in tumour where successive rounds of EMT and MET processes occur in response to cues from different environments.

It is thought that EMT and tumour dissemination are initiated at the advanced stage of tumour progression once tumour cells acquire the ability to invade following multiple successive rounds of mutation and selection for competitive fitness (Cairns 1975). This prevailing dogma has recently been challenged by an elegant animal model of pancreatic cancer progression in which lineage tracking was used to identify epithelial cells that have undergone EMT and disseminate to distant organs (Rhim, Mirek et al. 2012). In this study, EMT and cell dissemination to distant organs occur prior to the formation of an identifiable primary tumour. If dissemination and seeding of pancreatic epithelial cells precede the detection of tumour in humans, as they do in the mouse model, several waves of disseminated tumour cells may disseminate before diagnosis and may progress in parallel at different rates in different organs (Klein 2009).

Several GFs that act through receptor tyrosine kinases including (TGF β ; (Zavadil and Bottlinger 2005)) epidermal growth factor (EGF; (Lo, Hsu et al. 2007)), (FGF; (Lee, Dedhar et al. 2006), (Acevedo, Gangula et al. 2007)), (HGF; (Chaffer, Dopheide et al. 2007) and their downstream signal transduction pathways contribute to the loss of E-cadherin function and induce cancer cell migration. Other signalling pathways such as Wnt, Notch and Hedgehog pathways participate in EMT regulation (Smit and Peeper 2010). EMT can also be induced by

several oncogenes, including oncogenic Ras (Janda, Lehmann et al. 2002) ErbB2 (Jenndahl, Isakson et al. 2005), gain-of-function role of mutant p53 (Roger, Jullien et al. 2010), (Wang, Wang et al. 2009), (Kogan-Sakin, Tabach et al. 2011) and the neurotrophic receptor TRKB (Kupferman, Jiffar et al. 2010), (Smit and Peeper 2010). The tumour microenvironment participates in EMT regulation. The inflammatory cytokine interleukin-6 (IL-6) can promote EMT through JAK-STAT3 to activate Snail1 expression (Sullivan, Sasser et al. 2009).

Hypoxia condition, frequently existing in a tumour microenvironment can promote EMT by inducing expression of the transcription factor hypoxia-inducible factor 1 α (HIF1 α), which in turn activates the expression of Twist (Yang, Wu et al. 2008). EMT responses can be increased through crosstalk and cooperation between distinct pathways (Lamouille, Xu et al. 2014), (Savagner 2010).

Among the molecular alterations, these signalling pathways activate one or several transcription factors like Snail, Slug, ZEB1, ZEB2 and Twist that are master regulators of EMT. These transcriptional repressors of E-cadherin function during embryogenesis to convert epithelial cells to migratory mesenchymal cells and they are therefore essential for morphogenetic processes (Chen and Behringer 1995), (Nieto, Sargent et al. 1994), (Sefton, Sanchez et al. 1998). These transcription factors have distinct expression profiles according to the signalling pathways that initiate EMT and their contributions to EMT depend on the cell and tissue type. These transcription factors repress transcription of the E-cadherin gene, encoding an adhesion molecule important in the structure and strength of the epithelial sheets, and loss of E-cadherin has been detected in many invasive cancers (Batlle, Sancho et al. 2000), (Bolos, Peinado et al. 2003), (Yang, Chen et al. 2004). Cancer cells that have undergone EMT can break free from the primary tumour mass and move out as single cells and invade in to the surrounding tissue. However, many cancer types including breast, prostate and lung tumours, rhabdomyosarcoma and melanoma, retain cell-cell cohesive structures and may thus penetrate vessel walls collectively as cell strands (Ewald, Huebner et al. 2012), (Hegerfeldt, Tusch et al. 2002), (Parri and Chiarugi 2010), (Wolf, Wu et al. 2007). Therefore, the presence of E-cadherin does not preclude tumour cell invasion during tumour progression. The functional and clinical importance of EMT goes beyond the crucial initial step in cancer cell migration and invasion. EMT program play also a central role in other aspects of tumour malignancy by suppressing tumour apoptosis (see section apoptosis

(hallmarks) and senescence and by producing tumour stemness and immune evasion (Zheng and Kang 2014).

2-2 Cell migration plasticity and invasion

Cell motility is essential during embryogenesis, when cells are required to migrate to specific locations in the developing embryo in order to form various organs and limbs. Migration is initiated in response to a gradient of specific cues in the cell's environment. The nature of that cue often defines the type of directed migration. Cells will respond to chemotactic cues by moving toward a positive gradient of soluble stimulants (chemokines and GFs). Haptotactic migration is a response to a gradient of substrate-bound molecules. Haptotactic cues are similar to chemotactic, but they are subtler than a binary chemotactic cue. Mechanotactic migration results from changes tension of the physical surroundings, for example by wounding or changes in cell-cell adhesion. Electrotaxis is the response to an electric field and durotaxis is migration determined by ECM rigidity (Roussos, Condeelis et al. 2011).

In the healthy adult most cells are immotile, motility tends to occur only during immunity, wound healing and tissue regeneration. Cancer metastasis is arguably the most well-known manifestation of disordered cell migration and acquisition of a motile phenotype contributes to tumour progression. One of the crucial steps of acquiring invasive ability is the deregulation of signalling pathways leading to changes of gene expression and function that modulate the actin cytoskeleton dynamics. Alteration of actin cytoskeleton affects cell-cell contacts, cell adhesion and membrane protrusion which are all fundamental structures controlling cell motility (Olson and Sahai 2009).

2-2-1 Regulation of cell motility and invasion

The molecular pathways regulating invasion and motility and the specific mode of invasion employed by a cancer cell is largely cell-type specific in addition to the influence of the tumour microenvironment. Modulation of cell-cell contacts as well as cell matrix interactions is necessary for cancer cells to acquire a more migratory phenotype.

2-2-1-1 Rho GTPases regulation and functions in cell migration

The Rho GTPases family members Cdc42, Rac1 and RhoA regulate cytoskeleton organisation required for cell migration (**Figure 6**) and control other key cellular functions

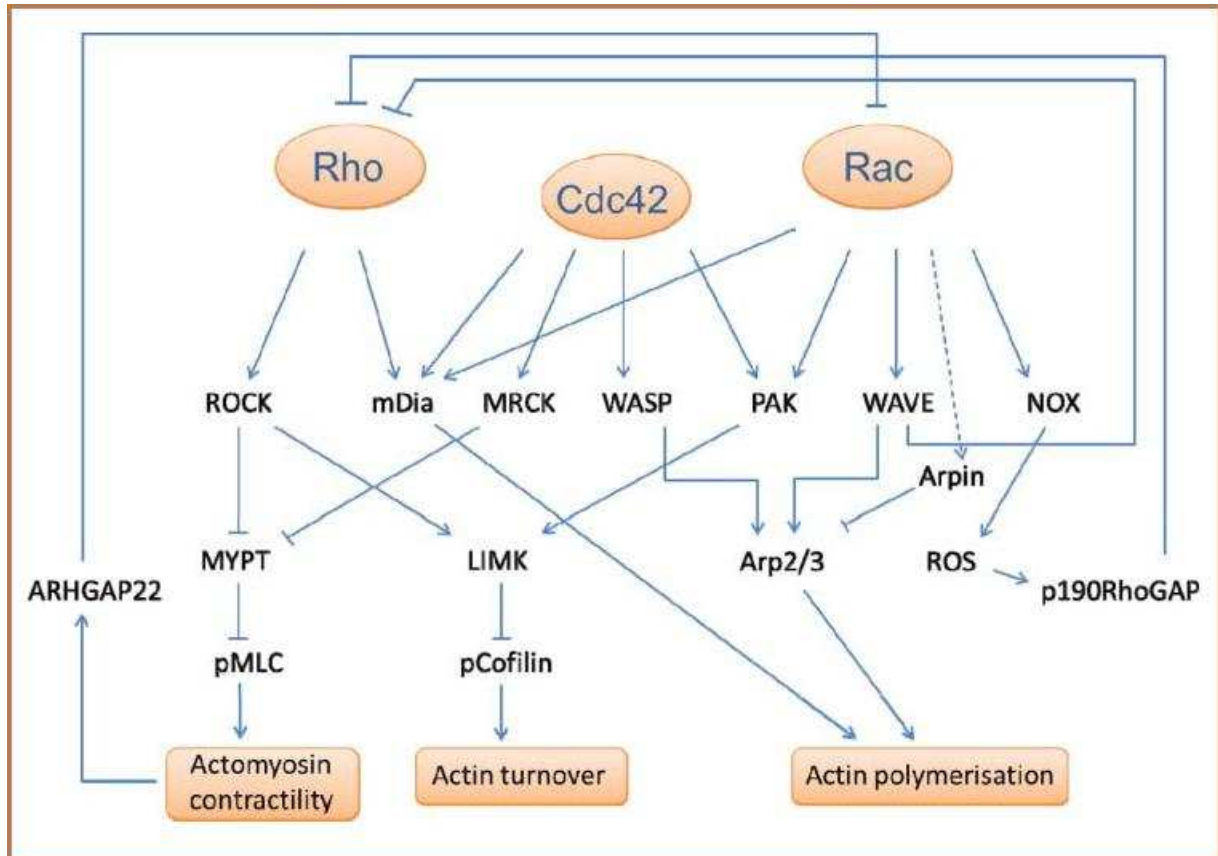


Figure 6. Crosstalk between classical Rho-family GTPases regulates actin remodelling during cell migration.

Rho promotes actomyosin contractility through Rho-associated protein kinase (ROCK)-dependent phosphorylation, and subsequent inhibition, of Myosin Light Chain (MLC) phosphatase MYPT. ROCK also phosphorylates LIMK, leading to inhibition of cofilin activity. Another effector of Rho is the formin mDia, which promotes actin polymerization during cell migration. Rho antagonizes Rac-mediated signalling through ROCK/contractility-dependent activation of the RacGAP ARHGAP22. Rac promotes actin polymerization and lamellopodia formation through activation of Wiskott-Aldrich syndrome protein family member (WAVE) and p21 Activated Kinase (PAK) and controls directionality through recruitment of Arpin, which inhibits WAVE-dependent activation of Arp2/3. Rac also antagonizes Rho-mediated signalling through a WAVE-dependent mechanism, and through activation of Nox-dependent ROS production, which promotes activation of p190RhoGAP, leading to inhibition of Rho. Cdc42 connects with both Rac and Rho, promoting actomyosin contractility through activation of MRCK, which phosphorylates MYPT and induces filopodia formation via WASP-mediated activation of Arp2/3.

Taken from (Sadok and Marshall 2014).

such as cell-cell adhesion, polarization, vesicle trafficking and cell cycle (Ridley 2006), (Orgaz, Herraiz et al. 2014), (Sahai and Marshall 2002). These GTPases act as molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form. The activity of Rho GTPases is tightly controlled by guanine exchange factors (GEFs) that promote GTP-loading. GEFs are activated in response to different extracellular signals including GF and cytokine receptors, integrins and cadherins (Buchsbbaum 2007).

As antagonists to GEFs, GTPase activating proteins (GAPs) inactivate Rho GTPases through their conserved catalytic GAP domain, which hydrolyses GTP to GDP. The upstream signals engaging RhoGAPs are poorly defined (Tcherkezian and Lamarche-Vane 2007). Rho GTPases are further negatively regulated by Rho guanine nucleotide dissociation inhibitors (GDIs), which bind the prenylated GDP-bound Rho proteins and prevent their translocation to the plasma membrane, thereby retaining Rho GTPases in inactive state and sequestered in the cytosol (Ren, Kiosses et al. 1999), (Boulter, Garcia-Mata et al. 2010). Tissue specificity of Rho GTPase effectors and distinct intracellular localizations of closely related Rho GTPases, due to different lipid modifications concur to determine specific Rho GTPase function (Etienne-Manneville and Hall 2002).

Spatiotemporal fine-tuning of Rho GTPases enables them to regulate signals in a transient and localized fashion, and such dynamic regulation ensures effective cell migration. Rho GTPases integrate intracellular signals downstream of mechano-sensors promoting cell protrusion formation and front-rear polarity, actomyosin contractility, and the turnover of cell-matrix and cell-adhesions, which jointly contribute to the type and efficacy of cell migration in moving cells. At least three types of cell protrusions occurring at the leading edge of migrating cells are regulated by Rho GTPases. Filopodia are thin membrane exploratory extensions that contain parallel bundles of actin filaments for mechanosensory sensing of the environment. Cdc42 is involved in filopodium formation. Cdc42 could bring together three of its target, the formin mDia2, WASP/N-WASP which nucleate and elongate actin filaments, and IRSp53, which bundles actin filaments (Ridley 2011), (Ridley 2015). Lamellipodia are sheet-like extension at the front of single cells or leading cells of collectively migrating cohort that provide adhesion to substrate, and are controlled by active Rac1, Cdc42, RhoA and RhoC (Ridley 2011), (Ridley 2015). Cdc42 plays a role in establishing cell migratory polarity, migratory persistence and promotes extension by

stabilizing the microtubule cytoskeleton (Etienne-Manneville and Hall 2001). Rac regulates branched actin network assembly and extension toward the leading edge through WAVE and Arp2/3 (Pollard and Borisy 2003), (Wu, Frey et al. 2009). Actin branching is further promoted by cofilin, which is activated downstream of the Rac-PAK-LIMK axis (Delorme, Machacek et al. 2007) or via RhoC-ROCK-LIMK (Bravo-Cordero, Magalhaes et al. 2013). Cofilin mediates the severing of existing cortical actin filaments at protrusions and thereby generates new barbed ends and hence new actin filaments, which enhances Arp2/3-mediated extension of lamellipodia (Bravo-Cordero, Magalhaes et al. 2013). Through PAK, Rac further supports integrin-mediated based adhesion to ECM and mechanical stabilization of forward protruding lamellipodia (Zegers 2008). As third principal protrusion type, membrane blebbing results from a two-step process of initial bleb-like membrane protrusion with secondary stabilisation of the bleb by the cortical actin network (Charras, Hu et al. 2006), (Paluch and Raz 2013). Membrane blebbing depends upon intracytoplasmic hydrostatic pressure, associated with high levels of active RhoA/ROCK signalling and downstream cortical actomyosin contraction (Vicente-Manzanares, Ma et al. 2009), (Poincloux, Collin et al. 2011), (Laser-Azogui, Diamant-Levi et al. 2014).

Besides cell protrusions at the leading edge, Rho GTPases control cell contractility at the trailing edge of moving cells. RhoA activates ROCK, which engages myosin light chain kinase and myosin II for actomyosin contraction, preferentially at lateral and rear cell portions (Vicente-Manzanares, Ma et al. 2009). Thereby, RhoA controls the retraction of the tail in migrating single cells and in cell groups, and likely, the lateral mechanocoupling toward cadherin-based cell-cell adhesions (Vicente-Manzanares, Ma et al. 2009), (Anon, Serra-Picamal et al. 2012).

In both Two-dimensional (2D) and Three-dimensional (3D) models for collective cell migration, Rho GTPases initiate and maintain leader and follower cell functions, interaction with substrate, cell-cell cohesion and supracellular coordination. At the leading edge of individually motile cells, Cdc42 and Rac1 regulate the actin cytoskeleton to form fingerlike filopodia and sheetlike lamellipodia, respectively, whereas in the cell body RhoA induces assembly of FAs and contractile actin-myosin stress fibers. To ensure each cell subregions to function simultaneously and in vicinity, cell subregions with active Rac show limited Rho activity, and vice versa, as consequence of reciprocal feed-back and mutual inhibition.

Downstream of Rac and Cdc42, Par6 and atypical PKC (aPKC) recruit the ubiquitin ligase Smurf1 to cellular protrusions, which degrades local levels of Rho, and thereby limits local Rho availability at the leading edge (Wang, Zhang et al. 2003). In addition, Rac activation leads to local inhibition of Rho by activating p190RhoGAP, either via direct interaction (Bustos, Forget et al. 2008), or indirectly, via engagement of Par6/aPKC (Hegerfeldt, Tusch et al. 2002), or p120catenin at cell-cell contacts (Wildenberg, Dohn et al. 2006). Likewise, the Rac effectors PAK1 and PAK4 promote the phosphorylation and inactivation of a set of RhoGEFs, which limits Rho activation (Zegers and Friedl 2014).

Conversely, Rho inactivates Rac either via direct engagement of the Rac inhibitors FilGAP (Ohta, Hartwig et al. 2006) and ARHGAP22 (Sanz-Moreno, Gadea et al. 2008) or via ROCK, which inhibits recruitment of the Cdc42/Rac GEF β PIX to cell-matrix adhesions (Kuo, Han et al. 2011). ROCK further inhibits the functional assembly of the polarity complex Par3/Par6, which limits Rac activation and thereby impairs proper leading edge formation and front-rear polarity (Nakayama, Goto et al. 2008). Thus, through negative crosstalk, potentially conflicting functions between Rho GTPases are distributed to and define functional subcellular compartments, including protrusions versus cell-cell junctions, apical versus basal zones, and actomyosin-dependent contractile versus actomyosin-independent regions in protrusions or cell-cell junctions.

2-2-1-2 Kinases and related molecules

Mesenchymal cells often rely on signals mediated by Src tyrosine kinase to acquire increased motility. Src is activated downstream of both RTKs and integrin adhesion receptors. It is a very potent oncogene with a variety of cellular substrates that elicit both transcriptional responses and regulate cell shape. For this reason, Src is able to induce both increased proliferation and survival of cancer cells, as well as promote motility and invasion. Together with FAK, Src affects Rho GTPase signalling by modulating GEF and GAP signalling downstream of integrins. For example, Src activates Rac1 at the leading edge by promoting activation of the GEF DOCK180 *via* recruitment of adaptor proteins Cas and Crk (Chodniewicz and Klemke 2004), *via* phosphorylation and activation of the Rac GEF Tiam1 or by phosphorylating Syk, which subsequently activates the Rac GEF Vav1 (Oberfell, Eto et al. 2002), (Servitja, Marinissen et al. 2003). Src is also able to transiently suppress RhoA

activity at the front of the cell by phosphorylation and activation of p190RhoGAP (Arthur, Petch et al. 2000). However, formation of podosomes, adhesive structures containing proteolytic enzymes, has also been shown to require Src mediated activation of RhoA (Berdeaux, Diaz et al. 2004). Src or FAK null cells show reduced migration and also have enlarged and stronger cell-matrix adhesions. Src/FAK signalling is therefore thought to promote motility by increasing FA turnover (Ezratty, Partridge et al. 2005), (Fincham and Frame 1998), (Webb, Donais et al. 2004). In addition, Src has been shown to induce expression of several MMPs. In fibroblasts, Src/FAK-mediated activation of Rac1 and JNK upregulates MMP9 levels and activates MMP2, resulting in enhanced invasion in Matrigel (Hsia, Mitra et al. 2003). Src can also induce EMT by deregulating E-cadherin, for example by disruption of its localization in KM12C colon cancer cells or inducing endocytosis of the E-cadherin complex in epithelial cells (Avizienyte, Wyke et al. 2002), (Fujita, Krause et al. 2002).

In line with these promigratory functions, an increased activity of the Rho family of monomeric GTPases and Src/FAK signalling is frequently encountered in many types of cancer cells (Parri and Chiarugi 2010). The Rho isoform RhoC is also important for contractility and motility and has been associated particularly with metastasis. For example in breast cancer cells, the MicroRNA miRNA-10b inhibits HOXD10 expression and controls a set of HOXD10 downstream genes leading to RhoC activation which promotes invasion. MicroRNA-10b is induced by the EMT-related transcription factor Twist1 (Ma, Teruya-Feldstein et al. 2007).

A crosstalk between JAK/STAT3 signalling and members of the Rho GTPase has now started to be evident in the context of malignant progression (Bournazou and Bromberg 2013). Rac 1 acting in a complex with MgcRacGAP (male germ cell Rac-GAP) promotes tyrosine phosphorylation of STAT3 upon cytokine stimulation and its nuclear translocation (Tonozuka, Minoshima et al. 2004). Cadherin engagement causes an increase in the levels and activity of Rac1 and Cdc42 protein, which in turn results in STAT3 activation (Raptis, Arulanandam et al. 2011). STAT3 is necessary for RhoA-mediated oncogenic transformation, and RhoA can activate STAT3 in a JAK2-dependent manner (Aznar, Valeron et al. 2001). A reciprocal activation is possible. Indeed, ROCK1 is activated by JAK1 in carcinoma-associated fibroblasts and ROCK1 promotes STAT3 phosphorylation (Sanz-Moreno,

Gaggioli et al. 2011). It seems that JAK1/2 and RhoA-ROCK1 signalling can be placed upstream and downstream of each other in different signal transduction pathways (Peelman and Tavernier 2013). Therefore, JAKs and ROCK may mutually enhance each other in tumour cells to propagate pro-tumourigenic signals.

2-2-2 Modes of cell migration

2D and 3D *in vitro* models, consisting of random or directed cell movement on 2D substrate and cell invasion towards chemo-attractants through a barrier into 3D matrices, have been performed both in combination with time-lapse or conventional microscopy methods to understand how cancer cells move. Cell migration refers to the act of the cell translocation in a 2D or 3D environment. Cell invasion is more precise in that it refers to a cell moving through a 3D matrix, dependently or independently of proteolytic mechanisms (Hulkower and Herber 2011). The development of two-photon or multi-photon intravital imaging microscopy allows the study of tumour cell invasion in living mouse models (Wolf, Wu et al. 2007). Human cancer studies reveal that cancer cells display different morphology and modes of invasion. They can migrate individually as single cells, as loosely attached cell streams and as well-organized adherent collective cells (**Figure 7**). The type of migration mode a cell uses will depend on its environmental context and the cell type. The migration of single cells has been divided into mesenchymal and amoeboid mode based on cell morphology, mechanism of force generation, cytoskeleton organization and characteristics of the cell-substrate interaction (Friedl and Wolf 2010).

2-2-2-1 Mesenchymal migration

The mesenchymal mode of cell motility either in 2D or 3D environment is a multi-step process that require the co-ordination of five steps which operate in a cycle and involves (1) actin polymerization form a locomotory protrusion at the leading edge; (2) integrin-mediated attachment of the protrusion to the ECM initiates focal contacts; (3) secretion of surface proteases to ECM contact-dependent ECM cleavage by surface proteases; (4) actomyosin contraction of the cell body to generate an elongated shape; and (5) rear retraction and translocation of the cell body allowing locomotion of the cell (Ridley, Schwartz et al. 2003), (Friedl and Wolf 2009), (Friedl and Alexander 2011).

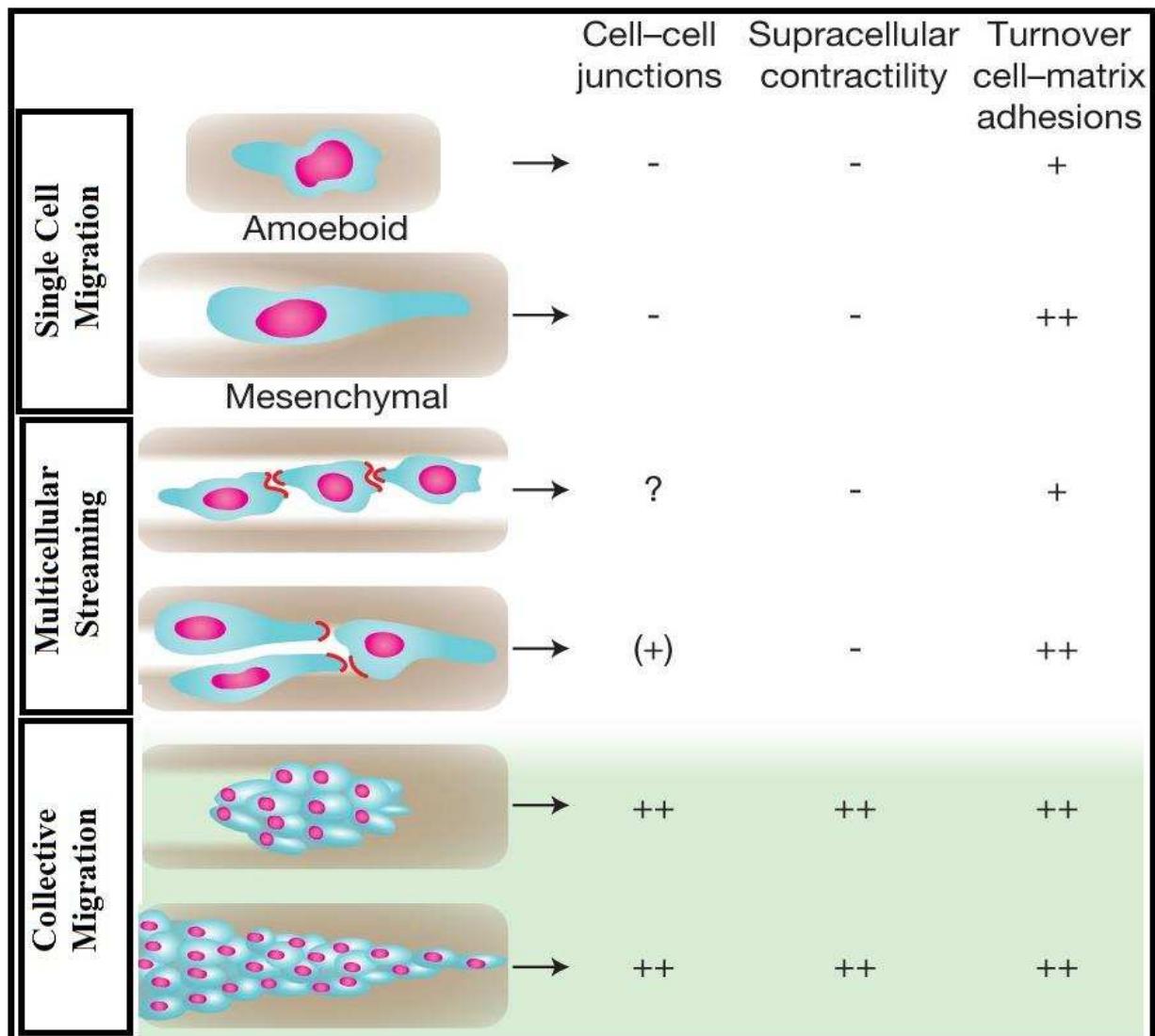


Figure 7. A schematic diagram representing the main modes of cancer cell migration.

These include: amoeboid or mesenchymal single-cell migration; multicellular streaming, and collective cell migration as small clusters or huge cohorts. Although each pattern can be described as a distinct process, overlapping or converting cell behaviour can result in mixed or unstable phenotypes. Intensity range of proposed properties (from absent to strong) is indicated by minus and plus signs.

Adapted from Friedl et al, Nat Cell Biol Rev 2012.

Mesenchymal motility is characterized by relatively slow migration speeds (0.1-1 $\mu\text{m}/\text{min}$), an elongated cell morphology and high adhesion-based traction (Friedl 2004). The mesenchymal migration requires an EMT and ECM proteolysis in order for the cell to create a pathway to migrate through the ECM. Mesenchymal movement is driven by a leading edge with the formation of cell protrusion in the form of filopodia and/or lamellipodia and utilizes dynamic actin polymerization, the branching of actin filaments confers intrinsic polarity. The cell-traction forces necessary for migration are propagated through the actin cytoskeleton via matrix integrin adhesions to enable actomyosin contraction and rearward retraction (Ridley, Schwartz et al. 2003), (Ridley 2001), (Berrier and Yamada 2007). Formation of actin-rich lamellipodia in response to GFs, cytokines and ECM components is Rac1 dependent in the mesenchymal mode of migration (Ridley, Schwartz et al. 2003), (Ridley 2001). The signal transduction through tyrosine kinases and G-protein-coupled receptors initiates the activation of Rac1 and production of phosphatidylinositol-3, 4, 5-triphosphate (PIP3), which are known to activate WASP/WAVE proteins and subsequently Arp2/3 complex. The Arp2/3 complex regulates spatiotemporal actin polymerization and lamellipod formation at the leading edge of the cell. The directionality of cell movement is maintained by Cdc42, which coordinates actin polymerization at the front of the cell with microtubule attachment and alignment (Ridley 2001), (Chou, Burke et al. 2003). The protruding leading edge is then stabilized by integrin interactions with the ECM and the formation of FAs that attach the new protrusion to the ECM. Some focal contacts develop into large FAs that enable the actomyosin contractility regulated by Rho and ROCK activity to be transmitted to the ECM, facilitating cell body to follow the leading edge and retract the cell rear (Sahai and Marshall 2003). The adhesion molecules lead to the accumulation of surface proteases, such as MMPs and uPA, that degrade ECM proteins providing space for the cells to crawl (Wolf, Wu et al. 2007). Although, mesenchymal migration is characteristic of fibroblasts, other cell types utilize this mode of migration including tumour cells from tumours of connective tissue (Friedl and Alexander 2011).

2-2-2-2 Amoeboid migration

Amoeboid cell migration is used by rounded cells such as hematopoietic stem cells, leucocytes and cancer cells (Friedl and Alexander 2011), (Lammermann and Sixt 2009),

which in a push and squeeze type of migration make their way through the ECM. Distinct amoeboid motility modes rely on different leading edge structure. The first form of amoeboid movement employ Rac-dependent actin rich filopodia at the leading edge and engage in weak attachment with the substratum and require no proteolytic ECM remodelling. The second type involves migrating cells with rounded morphology, forming blebs with very low adherence with the substratum and capable of propelling. The amoeboid motion is dependent on RhoA and ROCK activity (Sahai and Marshall 2003), where ROCK-dependent MLC phosphorylation is crucial for the correct organization of MLC and to generate traction forces within the moving cell (Wyckoff, Pinner et al. 2006). Phosphorylated MLC increases ATPase activity to promote actin-myosin interactions and contractile force generation. The intracellular pressure results in the rupture of the actomyosin cortex breaking the linkage between plasma membrane and cortical actin generating membrane blebs (Yoshida and Soldati 2006), (Paluch and Raz 2013). After the formation of the bleb, the contractile cortex re-assembles (Charras, Hu et al. 2006). Cells squeeze through the ECM only by mechanical force in the absence of significant proteolytic activity. Non-integrin adhesion mechanisms rather than specific integrin-mediated FAs are used, and thus no FA turnover is observed and the cells migrate rather fast (0.1-20 $\mu\text{m}/\text{min}$) (Friedl and Wolf 2003), (Wolf, Muller et al. 2003), (Wolf, Wu et al. 2007). Therefore, the amoeboid mode of migration requires synergistic coordination of morphological adaptation to ECM architecture and regulation of actomyosin-generated pressure for rapid cell motility (Lammermann and Sixt 2009), (Robbins, McGuire et al. 1999). However, the extent and type of collagen cross-linking seems to affect the requirement for proteolysis during amoeboid migration in vitro and in vivo, and the Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) proteolytic activity seems indispensable for amoeboid migration in naturally cross-linked collagen in vitro and in vivo (Sabeh, Shimizu-Hirota et al. 2009).

Cells invading in an amoeboid fashion require RhoA activity to induce a high level of actomyosin contractility to be able to squeeze through holes in the 3D matrix (Sahai and Marshall 2003), (Sanz-Moreno, Gaggioli et al. 2011). Known mechanisms leading to increased levels of active RhoA and activated ROCK include the loss of p53 (Guo and Zheng 2004), (Gadea, de Toledo et al. 2007), (Lefort, Mandinova et al. 2007), (Qin, Baudry et al. 2009). p53-null fibroblasts display enhanced formation of membrane blebs that can be suppressed by pharmacological inhibition of ROCK suggesting that RhoA is an important effector of p53 to promote the amoeboid cell migration (Gadea, de Toledo et al. 2007). RhoA

influences the localization of ezrin (Yonemura, Matsui et al. 2002) and inhibiting ezrin function impaired invasion only in rounded cells. In addition Smurf1, a ubiquitin ligase that targets RhoA for localised destruction at Rac-dependent protrusion promotes the rounded form of movement (Sahai, Garcia-Medina et al. 2007). Overexpression of EphA2 seems to be involved in the amoeboid mode of migration (Parri, Taddei et al. 2009). Little is known about extra-cellular signals and receptors regulating the amoeboid mode of migration.

2-2-2-3 Multicellular streaming

Multicellular streaming is characterized by loosely- or non-adherent individual cells, which move one after each other along the same path. A hallmark of this migration mechanism is that there is no supra-connection at the cytoskeletal level between cells; so the cytoskeleton of every cell acts independently to generate traction force on the matrix and cells establish weak adhesion. Cells in streams have typical speeds of 1-2 $\mu\text{m}/\text{min}$ and significantly longer and straighter paths compared to isolated migratory cells and can display amoeboid-like or mesenchymal phenotypes (Friedl and Alexander 2011).

2-2-2-4 Collective cell migration

Collective cell migration occurs when a group of cells migrate together. Cells move in a highly coordinated manner as interlinked multicellular files lead by one leader cell or as clusters, or sheets, which are multiple cells in diameter and lead by several leader cells (Friedl and Alexander 2011), (Alexander, Weigelin et al. 2013). Collective migration occurs during embryogenesis, development, angiogenesis and wound healing. In cancer, particularly carcinomas, collective migration occurs at the tumour border. High substrate stiffness enhances collective cell migration. Since often the tumour stroma is stiffer than normal tissue stroma, this may serve to encourage tumour invasion (Ng, Besser et al. 2012). Collective migration is typically the slowest mode of cancer cell migration (0.01-0.05 $\mu\text{m}/\text{min}$). Collective cell migration retains intact cell-cell junctions, a feature that is lost in single cell migration. In most cases of collective invasion, cells move simultaneously with a mechanism similar to a collective form of mesenchymal motility. Cells at the leading edge of the migrating sheet or strand form lamellipodia and pseudopodia protrusions and generate traction and contractility using FA and myosin-II. The rear of these cells maintains cell-cell contacts through E-cadherins and tight junction proteins and exerts pulling forces on the cells

behind them (Friedl, Locker et al. 2012), (Ng, Besser et al. 2012). Cells that migrate as clusters often behave in a cooperative manner where force and contractility is mediated via cell-cell junction that maintain the paracrine signalling making the cohort behave as one big cell (Friedl and Alexander 2011), (Hidalgo-Carcedo, Hooper et al. 2011). At the cell-cell junction actin fibers stabilize E-cadherin, but excess adhesion between cells can also block collective migration (Omelchenko and Hall 2012). Actomyosin contractility is thus tightly regulated at cell-cell contacts in collectively migrating cells. Silencing of RhoA inhibits the formation of cell-cell contacts while RhoA overexpression, depletion of the RhoGAPs myosin IXA or p190RhoGAP or depletion of RhoE, which antagonizes ROCK-driven actomyosin contractility, lead to cell scattering and impaired collective migration of different epithelial cells (Desai, Aryal et al. 2004), (Hidalgo-Carcedo, Hooper et al. 2011), (Omelchenko and Hall 2012).

A study in *Drosophila* border cells showed that increasing Rac1 activation over the basal level in just a single cell was able to direct the migration of the entire cell cluster (Wang, He et al. 2010), (Inaki, Vishnu et al. 2012). The signalling for collective migration is highly complex and uses a blend of intracellular signalling commonly found in single cell migration and signalling gradients throughout the cluster, which can determine migration behaviour (Bianco, Poukkula et al. 2007), (Ng, Besser et al. 2012). The leading cells of the collective cohort generate an invasion path to invade the ECM. The guiding cells use cell-matrix adhesion receptors such as β 1-integrin and local secretion of MT1-MMP at their leading edges to cleave collagen fibres and orient them in a way that generates microtracks into which the collective mass migration of follower cells can occur (Wolf, Wu et al. 2007), (Friedl and Wolf 2008). Collective cell migration and invasion is found in most epithelial and mesenchymal tumour types (Friedl and Gilmour 2009). Using 3D reconstitutions of serial tumour tissue slices a recent study show that human epithelial cancers invade in a predominantly collective fashion. Cells in invading cluster exhibit some alterations in EMT markers but single-cell migration seems to be absent (Bronsert, Enderle-Ammour et al. 2014). Collective invasion could give tumour cells a selective advantage where all clones with different properties could functionally cooperate in order to successfully metastasize. As such, a cluster of metastasizing tumour cells appears to act as one polarized entity (Friedl and Wolf 2003).

Clusters of Circulating tumour cells (CTCs) ranging from 2-50 cancer cells, also

called tumour microemboli are detected within the circulation of patients with metastatic cancers of different origin (Yu, Bardia et al. 2013), (Cho, Wendel et al. 2012) (Stott, Hsu et al. 2010). The presence of microemboli in the circulation of patients could arise from intravasation of small groups of collectively migrating tumour cells that maintain their cohesion. A recent study using a mouse breast cancer xenografts model suggests that tumour cell clusters in the circulation may indeed result from the entry of groups of cells that originate from single tumour, into the bloodstream, rather than intravascular aggregation of cancer cells following intravasation (Aceto, Bardia et al. 2014). It is currently not clear if migration mode influences later metastasis. However, the same study demonstrated that such tumour cells clusters display an increased propensity to colonize distant organs in reconstituted mouse models (Aceto, Bardia et al. 2014). Collective migration of grouped cells that maintain their cell-cell and cell-matrix connections may involve increased survival signals as CTC clusters circulate in the blood (Kim, Koo et al. 2012), (Hou, Krebs et al. 2012).

Besides using cell-autonomous mechanisms, collectively migrating cancer cells may also rely on the support of stromal cells to pave their way through the ECM. Indeed, leader cells on motion are identifiable in cell culture as they develop distinct morphological changes with a highly polarized cell shape and the induction of lamellipodia (Poujade, Grasland-Mongrain et al. 2007). These leader cells are connected by E-Cadherin cell-cell contacts to their neighbouring cells, and when dragging a column of cells behind them, open wound forming in the migrating cell mass are closed by cell proliferation. Moreover, in 3D co-culture system between squamous cell carcinoma cells and stromal fibroblasts, the leading cells are always fibroblasts and prepare migratory tracks for the migrating carcinoma cells by proteolytic digestion and by force-mediated remodelling of the matrix (Gaggioli, Hooper et al. 2007). This process involves Rho-mediated myosin light chain activity in fibroblasts and the activities of Cdc42 and myotonic dystrophy kinase-related CDC42-binding protein kinase in carcinoma cells. p53-depleted fibroblasts enhanced invasion of lung carcinoma cells in a 3D co-culture system and this process also requires direct contact between the two cell types (Otomo, Otsubo et al. 2014). Close proximity and probably direct contact between fibroblasts and carcinoma cells is required for invasion of epithelial cells that are retaining their epithelial organization. In contrast, collectively migrating highly invasive carcinoma cells (that have undergone EMT) or mesenchymal sarcoma cells are able to digest their way through the matrix without any support by stromal cells (Wolf, Wu et al. 2007).

2-2-3 Plasticity of tumour cell migration

Differentiated cell most often uses a pre-defined migration mode once they have acquired it. For example, leukocytes employ amoeboid migration, stromal cells move using mesenchymal and epithelial cells move in a collective manner. However, cells appear highly flexible in the ways and changes in composition of their microenvironment can induce adaptive switching between different migration modes of moving cells. This ability of cells referred as cell plasticity is an important factor in cancer metastasis, as disseminating tumour cells encounter a range of ECM geometries and composition during their escape from the primary tumour and their spreading to distant sites (Friedl and Wolf 2010), (Yilmaz and Christofori 2010).

Non-migrating cells such as epithelial cells undergo EMT that enables them to develop collective and/or individual migratory behaviours, illustrates the differentiation plasticity during cancer cells invasion and metastasis (Taddei, Giannoni et al. 2013). Extracellular signals engage the EMT process by inducing expression of multiple transcription factors guiding the achievement of the new plastic and adjustable phenotype. The transition from collective invasion to amoeboid (collective-to amoeboid transition (CAT)) movement relies on simultaneous weakening of cell-cell and cell-matrix interactions, i.e. disruption of E-cadherin mediated adherens junction and integrin-linked focal complexes (Wolf, Wu et al. 2007), (Friedl and Gilmour 2009). Melanoma cells move in cohorts of multicellular clusters but interfering with $\beta 1$ integrin function abolished these collective movements, and results in a switch from collective invasion to amoeboid single-cell migration style that does not require adhesion to the ECM (Hegerfeldt, Tusch et al. 2002), (Wyckoff, Pinner et al. 2006). Collective amoeboid migration can directly or *via* an intermediate mesenchymal migration step give rise to the amoeboid one, although the molecular mechanisms for dispersion remain unknown (Friedl 2004).

Microenvironment cues weakening cell-ECM interactions can convert tumour cells with a mesenchymal movement towards amoeboid migration (MAT). Inhibition of pericellular proteolysis using protease inhibitors, the weakening of integrin-ECM interactions by antagonists, the strengthening of RhoA/ROCK signal pathways can lead to MAT. The inhibition of matrix-degrading enzymes function required for mesenchymal motility fails to stop cancer cell invasion but leads to the switch to amoeboid motility (Wolf, Mazo et al.

2003). Blocking integrin function in mesenchymal cells leads to switch to an amoeboid movement (Friedl 2004), (Wolf, Mazo et al. 2003). Low adhesion and strong physical confinement, appears enough to trigger a mesenchymal to amoeboid switch, suggesting that tumour cells may spontaneously escape primary tumours and invade surrounding stroma without any specific genetic alteration (Liu, Le Berre et al. 2015). All the evidences imply that there exists a high degree of plasticity in cancer cell invasion and tumour cells adapt to different conditions by switching their properties and requirements (**Figure 8**).

In particular, the regulation of Rac activity appears to control the inter-conversion or plasticity between the mesenchymal and amoeboid modes of movement (Sanz-Moreno, Gadea et al. 2008). In melanoma cell, activation of Rac1 mediated by a complex containing the adaptor protein NEDD9 and the Rac1-GEF DOCK3 leads to the actin polymerization *via* WAVE2-Arp2/3 pathway directing the elongated movement. Activated Rac1 decreases phosphorylation of myosin light chain 2 through WAVE2, thus repressing amoeboid (Rho-mediated) movement. Conversely in amoeboid movement, activated RhoA signalling through ROCK signalling activates the Rac-GAP ARHGAP22, thus suppressing Rac activity and with it the mesenchymal motility (Sanz-Moreno, Gadea et al. 2008). Furthermore, tumour cells moving through all the obstacles to invade require various actin cytoskeleton organization. The versatile nucleating, elongating, capping, severing, crosslinking factors that regulate the actin assembly also provide a flexible platform of cytoskeleton reorganization favourable during migration and invasion. In N-WASP and WAVE2 depleted cells, mDia1 acts downstream of RhoA to form jagged protrusions with lamellar properties and to increase filopodia formation, indicating a crosstalk and compensatory mechanism between the two groups of actin nucleators in membrane protrusion formation required for cell motility (Sarmiento, Wang et al. 2008). Other pathways that lead to a shift of Rac/Rho balance in favour of Rho activity induce MAT. The interference with Rab-5 mediated endocytosis and recycling of active Rac to cell protrusion downregulate Rac activity (Palamidessi, Frittoli et al. 2008). The inhibition of E3 ubiquitin ligase Smurf1, which leads to Rho degradation and down-regulation of ROCK directly at the cell periphery grants for dominance of Rac at the front of polarized cells (Sahai, Garcia-Medina et al. 2007).

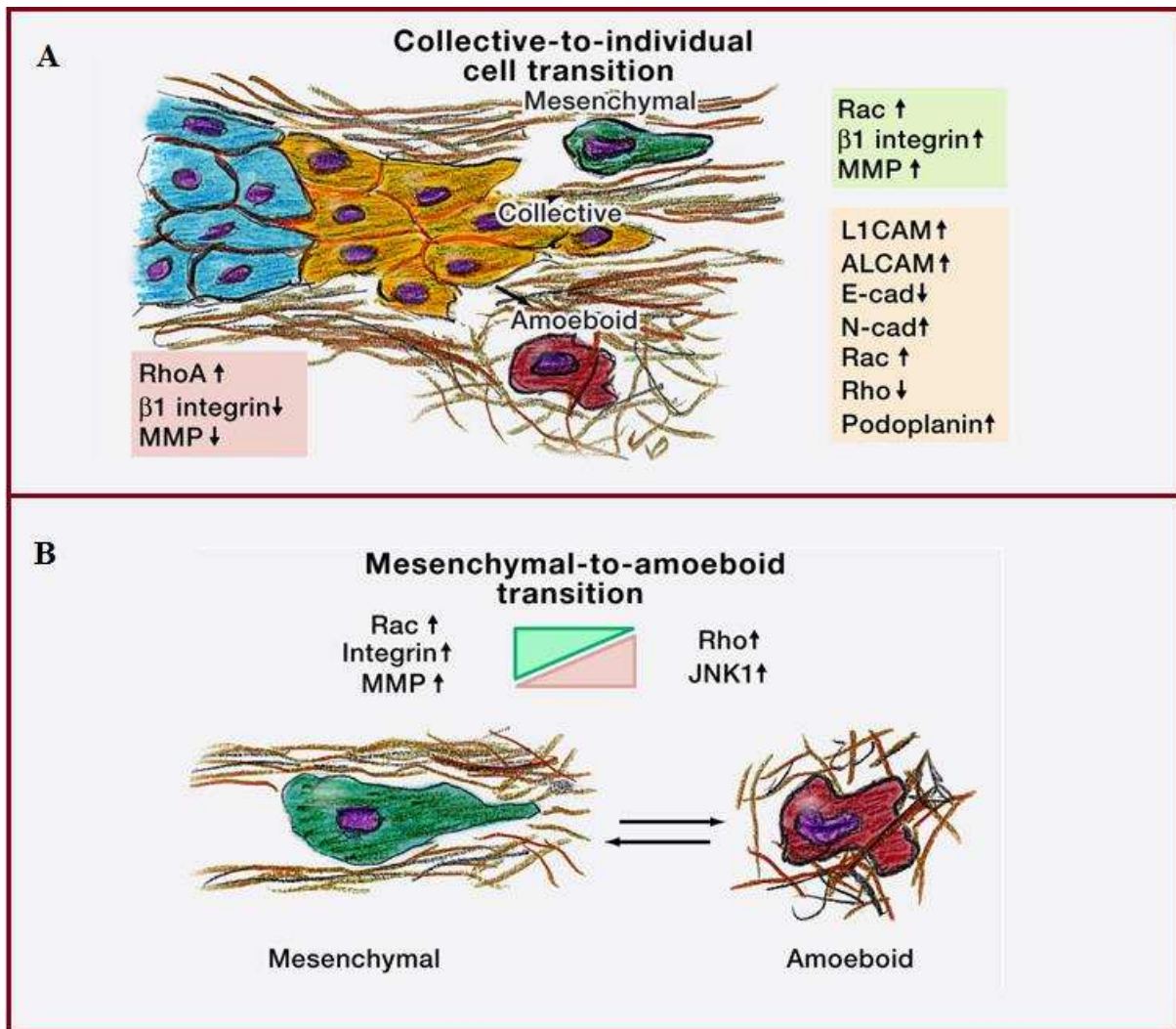


Figure 8. Plasticity of tumour cell invasion.

Invasion programs display plasticity, or adaptability, including transition from collective cell migration to individual cell migration (C) and mesenchymal to-amoeboid transition (D). Key regulators of these transitions altered in expression or function are indicated.

Taken from (Friedl and Alexander 2011).

2-3 Matrix remodelling

In order to escape the primary tumour and reach the blood vessels, cancer cells need to push away obstacles and dig passageways through the ECM of the nearby tissues. The ECM is remodeled through rearrangements of matrix components by cleavage and deposition. For this purpose, cancer cells use different matrix-degrading proteases, secreted either by themselves or by stromal constituents like fibroblasts, macrophages and mast cells (**Figure 9**). The MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families are the main enzymes responsible for matrix degradation. Enzymes involved in remodelling are regulated at the transcriptional level and by ensuring proper localization at the cell surface. MMPs are also controlled by tissue inhibitors of metalloproteinases (TIMPs) and many MMPs are synthesized as inactive pro-enzymes that need to be activated by other proteases, often other MMPs. In addition, MMPs can either be membrane-bound or elicit their function as secreted soluble proteins. Despite the tight control of MMP activity, these enzymes are frequently overexpressed and activated in malignant tissues (Kessenbrock, Plaks et al. 2010). MMPs cleave collagens, proteoglycans, fibronectin, laminin and tenascin of the ECM to make way for the cells to move and they also mobilize previously inactive GFs from the ECM to the benefit of the tumour cells (Tatti, Vehvilainen et al. 2008) (**Figure 9**). Collagen I can be cleaved by the soluble MMP-1, MMP-8 and MMP-13 enzymes, but also by the transmembrane MMP14 (MT1-MMP). Active MT1-MMP at the cell-matrix interface cleaves intact fibrillar collagen that acts as barriers to migration. Non proteolytic migration is either maintained by amoeboid cell deformation (Wolf, Mazo et al. 2003) or is inhibited (Sabeh, Ota et al. 2004), dependent on the type of collagen scaffold used as migration substrate (Packard, Artym et al. 2009), (Sodek, Brown et al. 2008), (Sabeh, Shimizu-Hirota et al. 2009). In addition to collagen I, MT1-MMP also degrades collagen II and III, fibronectin and laminin and is able to activate pro-MMPs. MT1-MMP was also shown to be needed for invasion of cancer cells through reconstituted basement membrane (Hotary, Li et al. 2006) and MT1-MMP overexpression in normal epithelial cells leads to increased formation of locally invading tumours (Soulie, Carrozzino et al. 2005). Studying cell-matrix interactions *in vitro* has been done on both 2D surfaces where cells are adhering to a matrix coat and in 3D conditions where cells are embedded into the matrix. Differences in matrix stiffness and architecture can influence intracellular signalling pathways and examining these pathways under more *in vivo*-like 3D conditions is therefore becoming increasingly important. Examples of such assays are the Boyden chamber migration and invasion assays, gel

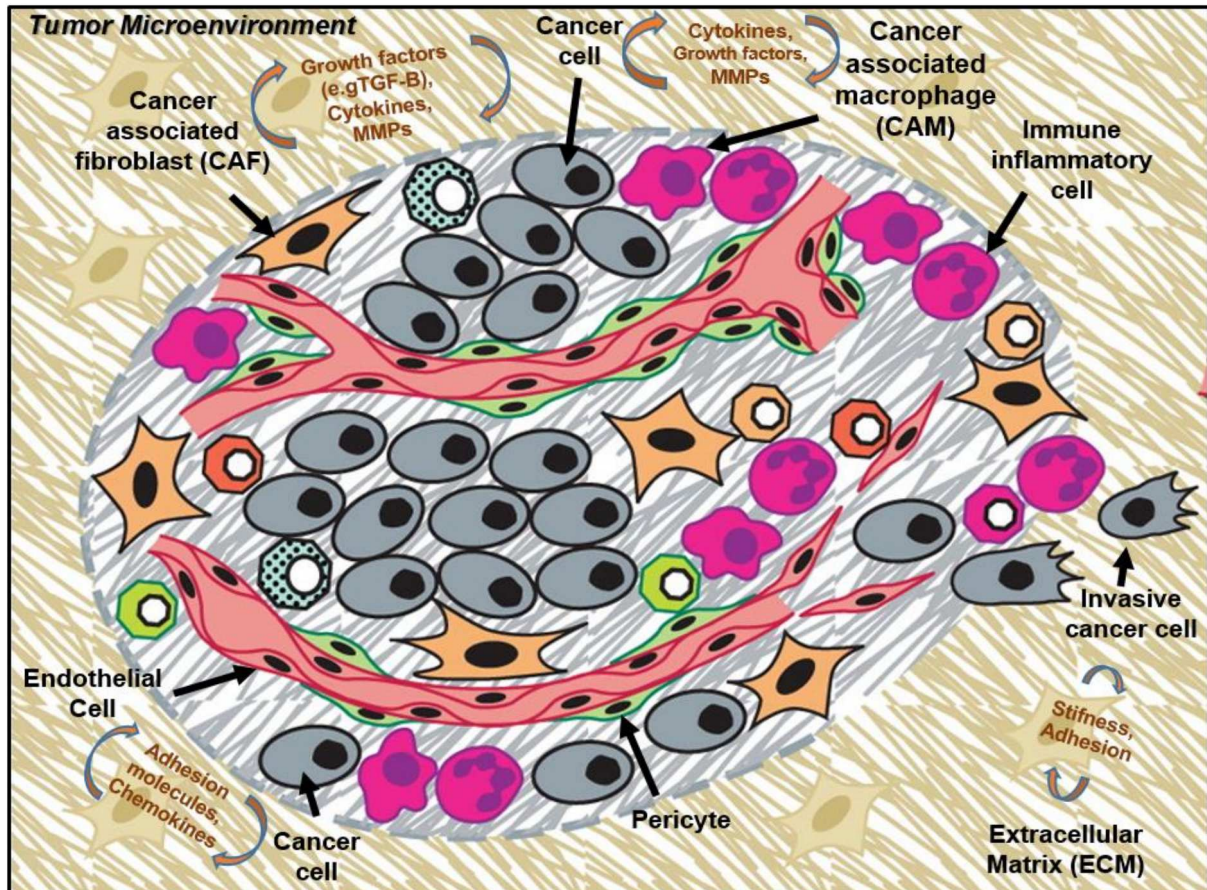


Figure 9. Interactions between cancer cells and tumour microenvironment.

An assemblage of distinct cell types constitutes most solid tumours. Both the parenchyma and stroma of tumours contain distinct cell types and subtypes interact with cancer cells. Those interactions are mediated by direct cell-to-cell contact and/or release of cytokines, chemokines, GFs, matrix metalloproteases (MMPs), and ECM proteins and collectively enable tumour growth and progression.

Adapted from (Hanahan and Weinberg 2011)

contraction assays and organotypic models where a mixture of different matrix constituents and stromal cells are used. 3D matrices consist of ECM components like collagen-I or matrigel, a dense network of primarily collagen-IV and laminin similar in composition to the basement membrane (Kleinman and Martin 2005). The properties of collagen-I networks like fibre thickness, pore size, mechanical properties depend on the polymerization conditions and can therefore be experimentally controlled (Wolf, Te Lindert et al. 2013).

In floating collagen assays, fibroblasts embedded into the matrix adhere to the collagen fibres and contract the network to a denser structure. The ability of cells to exert mechanical force and contract the matrix reflects their ability to remodel it (Gaggioli, Hooper et al. 2007). In fibroblasts, PDGF has been shown to induce matrix contraction via activation of ROCK and PAK1 (Rhee and Grinnell 2006). In contrast, lysophosphatidic acid (LPA)-induced contraction was not dependent on ROCK, but instead induced co-operation of PAK1 with the Rho effector mDia1 (Rhee and Grinnell 2006). PAK1 silencing led to reduced membrane ruffling and matrix contraction. Using function blocking antibodies or knock-out cells, it has also been shown that collagen binding integrins $\alpha 2\beta 1$ and $\alpha 11\beta 1$ are important for contraction of collagen I (Langholz, Rockel et al. 1995), (Svendsen, Barczyk et al. 2009). Some carcinomas have a limited ability to remodel the matrix and invade and instead hijack stromal fibroblasts to do the job for them (**Figure 9**). Cancer cell-derived GFs and cytokines can promote integrin $\alpha 5$ -mediated activation of ROCK, as well as activation of JAK1 and induction of pSTAT3 in stromal cells, thus converting them into so called cancer-associated fibroblasts (CAFs).

These CAFs are able to contract collagen and also make passageways in the surrounding matrix by JAK1-ROCK- controlled actomyosin contractility, thereby enabling cancer cell invasion (Cirri and Chiarugi 2011), (Gaggioli, Hooper et al. 2007), (Sanz-Moreno, Gaggioli et al. 2011). A chemical screen identifying compounds that inhibit collagen gel contraction also revealed a role for the small GTPase Rab21 in this process. Rab21-mediated delivery of integrin $\alpha 5$ to the plasma membrane was needed for induction of RhoA-mediated contractility (Hooper, Gaggioli et al. 2010). In addition to just clearing tracks in the ECM, matrix remodelling can also facilitate migration of cancer cells by modifying the orientation of matrix fibres. In breast tumours, collagen I is often linearized rather than deposited as relaxed fibrils and this could facilitate carcinoma migration along the matrix (Levental, Yu et al. 2009), (Wolf, Wu et al. 2007) (**Figure 9**). Cells with fast-migrating mode align their

protrusions parallel to large collagen fibres, using the fibres as tracks for directional migration (Alexander, Weigel et al. 2013), (Geraldo, Simon et al. 2012). Cells likewise sense the stiffness of the matrix, which is to a large extent defined by its architecture. The force exerted by the matrix on the cells, and vice versa, is subsequently mediated to the interior of the cell and ultimately determines gene expression and cell behaviour (Butcher, Alliston et al. 2009), (Zaman, Trapani et al. 2006). In the context of breast cancer progression, breast cancer stroma is often stiffer than normal tissue due to increased collagen deposition or cross-linking, or reduced turnover (Butcher, Alliston et al. 2009), (Paszek, Zahir et al. 2005). Increased collagen cross-linking can lead to increased integrin-mediated signalling and motility and is often due to excess activity of the enzyme lysyl-oxidase (LOX) secreted by tumour cells themselves in response to hypoxia (Baker, Bird et al. 2013), (Levental, Yu et al. 2009).

CHAPTER 3. THE PLASMINOGEN ACTIVATOR INHIBITOR, TYPE-1 (PAI-1) AND ITS ROLE IN CANCER INVASION

The breakdown of matrix macromolecules is catalysed by proteases released from the invading cancer cells. Most of these proteases belong to one of two main classes: matrix metalloproteases (MMPs) (Parsons, Watson et al. 1997), (Pepper 2001) or serine proteases (Andreasen, Kjoller et al. 1997), (Andreasen, Egelund et al. 2000). Proteases give cancers the ability of malignant cells to break out of tissue compartments. They process also GFs, membrane-associated receptors, a large variety of ligands, and proenzymes (Overall and Blobel 2007). Due to the large variety of substrates they target, extracellular proteases have an extremely complex role in cancer and have been shown to have an anti-tumourigenic as well as a pro-tumourigenic function. Adding to the complexity of their function is the fact that natural inhibitors, which often have other functions besides the control of extracellular proteolysis, tightly regulate their activity. The enzymatic system we will focus on in this chapter is the urokinase Plasminogen Activation System (uPA System), which consists of: uPA, its receptor uPAR, the urokinase plasminogen activator receptor, plasmin, the matrix degrading enzyme and PAI-1 (Blasi and Sidenius 2010), (Dass, Ahmad et al. 2008), (McMahon and Kwaan 2008).

3-1 Overview of urokinase plasminogen activator system

uPA is an extracellular serine protease. Cells secrete its enzymatically inactive form pro-uPA into the extracellular space. pro-uPA is activated by plasmin to its active form uPA. uPA concentrates at the cell membrane where it becomes activated upon binding to uPA receptor (uPAR). uPA has restricted substrate specificity, its only identified biological substrate being the pro-enzyme plasminogen which it converts to enzymatically active form plasmin. Plasminogen is a ubiquitous protein produced mainly in the liver and present in blood and the matrix. Plasmin itself is a broadly acting serine protease that, either directly or through the activation of other proteases, catalyses the breakdown of many of the known ECM and basement membrane molecules, such as Vitronectin, fibronectin, fibrin, laminin, thrombospondin, and collagens. Plasmin also activates pro-metalloproteases and converts pro-GFs into their active forms (Dano, Behrendt et al. 2005), (McMahon and Kwaan 2008). Therefore, to maintain tissue homeostasis and to avoid unrestrained tissue damage, the process of plasminogen activation in a healthy organism is strictly controlled through the availability of uPA, localised activation, and interaction with specific physiological inhibitors

(PAIs). One of these inhibitors, PAI-1, is believed to be the most abundant fast-acting inhibitor of uPA *in vivo* (Andreasen, Kjoller et al. 1997), (Andreasen, Egelund et al. 2000). Indeed, once secreted uPA is associated with PAI-1, uPA remains inactive. PAI-1 has a high affinity to the matrix constituent Vitronectin and Vitronectin-bound PAI-1 remains in an active conformation for extended periods of time. These facts suggest that uPA system has a multi-functional role in cancer biology. The system seems to function not only in cancer cell migration and invasion, but also in remodelling of the tissue surrounding the cancer cells, which may contribute decisively to the overall process of metastasis.

3-2 PAI-1 in regulation of cell adhesion, migration and invasion *in vitro*

3-2-1 General features of PAI-1

PAI-1 is a major inhibitor of the uPA-uPAR system. It is widely expressed in many cell types, such as fibroblasts, smooth muscle cells, endothelial cells, hepatocytes, platelets, and many different types of cancer cells (Saksela and Rifkin 1988), (Zorio, Gilibert-Estelles et al. 2008), (Cesari, Pahor et al. 2010). PAI-1 regulates plasmin function during fibrinolysis and ECM remodelling. Plasmin, a broad-spectrum serine protease, degrades fibrin and ECM proteins by which it plays a central role in clot dissolution and extracellular remodelling. Plasminogen activators (PA, tissue-type or urokinase-type) are required to convert the inactive proenzyme plasminogen to active plasmin by cleavage at a single arginine-valine bond (Kruithof, Tran-Thang et al. 1984). While tissue-type plasminogen activator (t-PA) mainly regulates intravascular plasmin activity through binding to its receptor annexin II on the endothelial cell surface, uPA acts by binding to its cell membrane receptor, uPAR, to promote plasmin induced pericellular proteolysis and to modulate cell migration (Czekay and Loskutoff 2004), (Myohanen and Vaheri 2004), (Kwaan and McMahon 2009). The main endogenous inhibitors of uPA and tPA catalytic activity are PAI-1, also called as SerpinE1 (serine protease inhibitor, clade E, member 1), and PAI-2 (SerpinB2) (Kruithof 1988). PAI-1 and PAI-2 both belong to the serpin superfamily of serine proteases inhibitors (Lowell 2006). **(Figure 10).**

PAI-1 is a secreted single-chain glycoprotein with 379 amino acids (about 52 KD protein) and forms a 1:1 complex with tPA and uPA. PAI-2 circulates as a 60 kD secreted

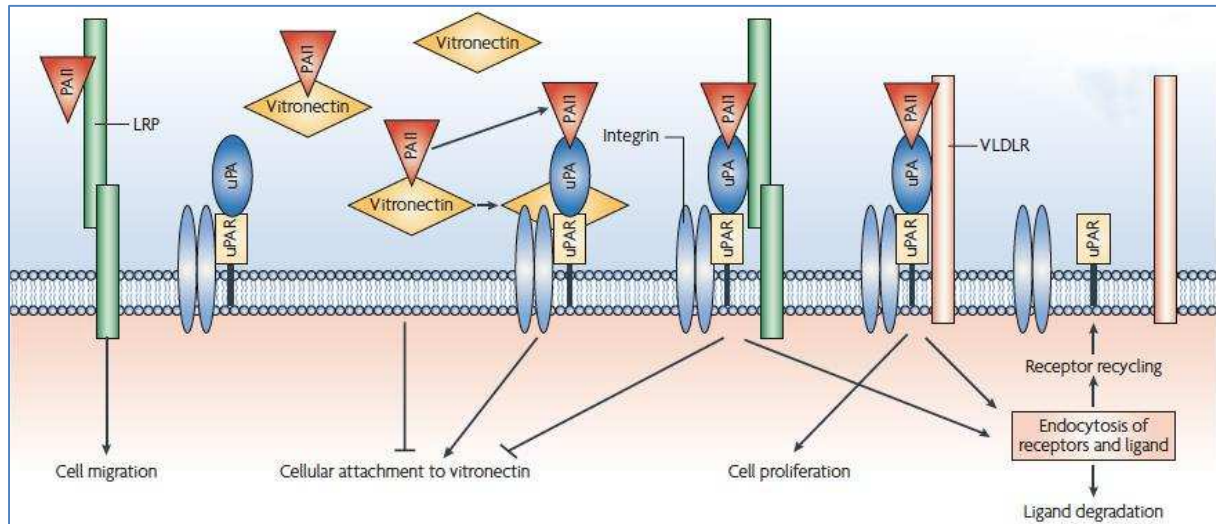


Figure 10. An overview of PAI-1 interactions with other plasminogen activation system components and their relevant biological roles.

PAI1 contributes to poor patient prognosis through the stimulation of tumour vascularization, growth and metastasis. This is achieved through various complex interactions that increase both cell proliferation and migration. PAI1 bound to Vitronectin prevents cellular attachment through uPAR and integrins. However, on uPA inhibition, PAI1 loses its affinity for Vitronectin, freeing up Vitronectin for binding by the now co-localized uPAR and integrins, initiating the rounds of cell attachment and de-attachment required for efficient cell migration. Following uPA inhibition, uPA–PAI1 binds with high affinity to members of the low-density lipoprotein (LDL) receptor family, stimulating endocytosis, degradation of uPA–PAI1, and partial recycling of the receptors. However, this interaction also generates other cell type- and receptor specific responses. The interaction of uPA–PAI1 with Low-Density Lipoprotein Receptor-Related Protein (LRP) causes a loss in cell adhesion due to the removal of integrins from the plasma membrane. The interaction of uPA–PAI1 with very-low-density lipoprotein receptor (VLDLR) increases cell proliferation. Additionally, PAI1 can bind directly to LRP, inducing activation of the JAK–STAT pathway, leading to increased cell motility.

Taken from Jan K. Jensen, Biochemical Journal 2006.

glycosylated and a 47 kD non-glycosylated intracellular form, though both forms are derived from the same mRNA and perform similar physiological functions. Of these two inhibitors, PAI-1 is the more rapidly acting, being 10- to 100- fold faster than PAI-2, at least *in vitro* (Thorsen, Philips et al. 1988) and is more widely expressed throughout the tumour than PAI-2 (Schaller and Gerber 2011), (Dass, Ahmad et al. 2008), (Lee, Cochran et al. 2011). By controlling the PA-plasmin proteolysis cascade, PAI-1 is crucial to maintain haemostasis and many other biological processes that involve the remodelling of the ECM (Sprengers and Kluft 1987), (Loskutoff, Sawdey et al. 1989), (Dellas and Loskutoff 2005). PAI-1 is evolutionarily conserved and found in many animal species, including human and mouse (Ginsburg, Zeheb et al. 1986), (Prendergast, Diamond et al. 1990).

3-2-2 PAI-1 activity changes via active/latent conversion

PAI-1 contains conserved structures for inhibiting proteinases that serves as a pseudo substrate of plasminogen activators (Sancho, Declerck et al. 1995). PAI-1 is either active, latent or cleaved, where only the active conformation can bind and inhibit uPA or tPA to inhibit plasmin-mediated proteolysis (Schroeck, Arroyo de Prada et al. 2002). Most of PAI-1 is bound to the ECM protein Vitronectin (Madsen and Sidenius 2008), and has been found to induce the multimerization of Vitronectin (Seiffert and Loskutoff 1996). Vitronectin binding to PAI-1 maintain PAI-1 in its active conformation and extends its half-life, while free active PAI-1 is rapidly converted to the latent form (Zhou, Huntington et al. 2003). PAI-1-Vitronectin interaction does not affect the inhibitory effect of PAI-1 on PAs or the activity of Vitronectin on cell adhesion (Lindahl, Sigurdardottir et al. 1989). Additionally, by interacting with Vitronectin, PAI-1 restricts pericellular proteolysis at focal contact sites (Salonen, Vaheri et al. 1989). Binding of active PAI1 to uPA-uPAR trigger the internalization of the complex in an LRP-1-dependent manner. PAI-1 and uPA are degraded in lysosomes while uPAR and LRP1 receptors are recycled back to the cell surface for another round of binding (Herz and Strickland 2001). The three different conformations of PAI-1 can bind LRP1. LRP1 is a large endocytotic receptor that can bind to over 30 different ligands (Lillis, Mikhailenko et al. 2005), a function that explains its involvement in numerous cellular processes, including cell migration and invasion (Dedieu, Langlois et al. 2008), (Langlois, Perrot et al. 2010), (Garg, Goyal et al. 2010).

It was initially thought that PAI-1 could function as a good anticancer drug, by

inhibiting proteolytic activity (Soff, Sanderowitz et al. 1995), (Stefansson, Petitclerc et al. 2001), (Bajou, Noel et al. 1998), and it was therefore surprising to find that high levels of stromal PAI-1 conveyed poor rather than a favourable outcome in several types of cancer; breast cancer (Janicke, Schmitt et al. 1993), (Grondahl-Hansen, Christensen et al. 1993), (Foekens, Schmitt et al. 1994), (Duffy, McGowan et al. 2014), colorectal cancer (Chen, Peng et al. 2015), gastric cancer (Ding, Zhang et al. 2013), pulmonary adenocarcinoma (Pedersen, Grondahl-Hansen et al. 1994) (Bayramoglu, Gunes et al. 2014), ovarian cancer (Kuhn, Pache et al. 1994), (Chen, Tai et al. 2014) and oral cancer (Magnussen, Rikardsen et al. 2014).

3-2-3 PAI-1 forms: Free or bound to matrix

PAI-1 is a secreted microenvironmental protein that is found either in its soluble or matrix-bound protein (usually to Vitronectin). Experimentally, PAI-1 biological functions were generally studied using PAI-1 in solution or free form. Its matrix-bound form function was not eventually determined and assumed to be identical to the soluble form. However, a dual role for matrix-bound and soluble forms of PAI-1 has been proposed in a model to study the restoration of alveolo-capillary barrier integrity. PAI-1 soluble form as an inhibitor of proteolysis and the matrix bound form to be involved in the regulation of cell migration (Maquerlot, Galiacy et al. 2006). Furthermore, PAI-1 matrix bound form was reported to maintain the blebbing of invasive SW620 colon carcinoma cells, thus promoting Mesenchymal-Amoeboid Transition (MAT) (Cartier-Michaud, Malo et al. 2012), (Malo, Charriere-Bertrand et al. 2006). However, the addition of soluble PAI-1 in solution to those experimental conditions did not have a significant effect on cell blebbing, indicating a differential role between the two forms (Cartier-Michaud, Malo et al. 2012).

3-2-4 Modulate ECM remodelling

PAI-1 also modulates ECM remodelling. During tissue remodelling, proteolysis is required to degrade the ECM barrier to allow cell movement. Plasmin plays an active role in remodelling the airway and vascular systems (Lijnen 2001), (Kucharewicz, Kowal et al. 2003), (Rakic, Maillard et al. 2003). In contrast to uPA, plasmin is broad-spectrum protease, which directly degrades most glycoproteins and can activate the proenzymes of MMPs, such as MMP1 and MMP3 (Deryugina and Quigley 2012). These activated MMPs in turn can degrade the diverse forms of collagen, kallikrein-related peptidases, and other proteins in the

ECM (Lijnen 2001). Thus, the uPA-mediated generation of plasmin creates a powerful proteolytic system capable of remodelling ECM. In addition, pericellular proteolysis also promotes the release of bioactive fragments and GFs from the ECM (Deryugina and Quigley 2012), which in turn, regulates cell proliferation and cell migration.

Plasmin-mediated proteolysis is positively regulated by uPA. A property of uPA that differentiates it from most other proteases is that it function while bound with high affinity to its receptor, uPAR. uPAR is a glycosyl phosphatidyl inositol (GPI) - anchored protein that tethers uPA on the cell surface to regulate pericellular proteolysis (Blasi, Vassalli et al. 1987), (Vassalli, Sappino et al. 1991), (Ossowski and Aguirre-Ghiso 2000). As uPAR lacks a transmembrane domain and it is unable to directly initiate a downstream signalling. In order for such signalling to occur, uPAR must interact with adjacent signalling molecules such specific integrins, leading to cell signalling through FAK, Src, Ras-MAPK, ERK and the Rho family small GTPase Rac (Smith and Marshall 2010). These signalling systems regulate cell proliferation and cell migration. uPA thus may trigger cell signalling by two distinct mechanisms either by directly binding to its receptor uPAR or indirectly by activation of plasmin which releases GFs sequestered in the ECM.

PAI-1 helps to maintain the integrity of the ECM in three ways. First, PAI-1 directly blocks uPA activity and constrains the proteolytic activity of plasmin. Second, it reduces the uPA level on the cell surface by promoting LRP mediated endocytosis (Degryse, Sier et al. 2001). Third, elevated PAI-1 can cause accumulation of fibrous material, which promotes fibrosis (Loskutoff and Quigley 2000), (Kucharewicz, Kowal et al. 2003).

3-2-5 Modulates cell attachment and migration

Cell migration occurs during normal physiological processes such as embryo morphogenesis, and during pathological processes such as wound healing, inflammation and cancer metastasis. Cell migration requires a series of dynamic motions including protrusion in the front, adhesion to the ECM or adjacent cells, detachment and retraction in the back (Ridley, Schwartz et al. 2003). PAI-1 plays a role in regulating directional cell movements. This is demonstrated by the following observations. PAI-1 expression is increased in keratinocytes at the wound margin and is deposited into the migration tracks of these cells

during the healing of monolayer wound (Providence, Higgins et al. 2008). Addition of active PAI-1 in solution promotes directional migration of both wild type and PAI-1 deficient (PAI-1, -/-) keratinocytes (Providence, Higgins et al. 2008). Treating cells with PAI-1 neutralizing antibody significantly slowed wound closure (Providence, Higgins et al. 2008). It has also been reported that PAI-1 controls uPA-mediated cell migration by balancing cell adhesion and detachment activities (Deng, Curriden et al. 1996). PAI-1 modulates cell migration in two opposing ways. On the one hand, PAI-1 inhibits smooth muscle cell movement by inhibiting cell adhesion (Stefansson and Lawrence 1996). On the other hand, PAI-1 stimulates migration of a variety of cells, including rat smooth muscle cells, highly invasive human fibrosarcoma HT-1080 cell, and non-invasive human melanoma IF6 cells (Degryse, Sier et al. 2001). PAI-1 recruits interstitial macrophages and myofibroblasts, which are key cellular factors in fibrosis (Oda, Jung et al. 2001).

3-2-5-1 PAI-1 modulates cell migration through regulating pericellular proteolysis.

Plasmin mediated proteolysis removes the tissue barrier that prevents cell migration (Allan and Martin 1995). Plasmin also promotes cell migration by activating cell signalling pathways such as p38MAPK/Akt2 pathway (Li, Syrovets et al. 2010). A high level of PAI-1 inhibits cell migration by blocking cell surface proteolysis by plasmin (Andreasen, Egelund et al. 2000). Meanwhile, a low concentration of PAI-1 is required for blocking excessive plasmin mediated-degradation of the ECM to maintain the matrix scaffold required for cell migration. Therefore, PAI-1-dependent preservation of the surrounding matrix facilitates motility of invading cells. Because both the plasminogen activation system and PAI-1 are involved in cell migration, the balance between PAs (t-PA and u-PA) and PAI-1 plays a crucial role in modulating the cell migration (Booth, Simpson et al. 1988).

3-2-5-2 PAI-1 inhibits uPAR-integrin mediated cell adhesion and regulates cell detachment.

The expression of the Vitronectin receptor, integrin and PAs is enhanced under circumstances requiring ECM remodelling, such as wound healing (Jang, Tsou et al. 2000), (Noszczyk, Klein et al. 2002). uPAR forms a stable complex with integrins on the cell surface, which promotes cell binding to Vitronectin through uPAR in the ECM (Wei, Lukashev et al. 1996). With a relatively high affinity to Vitronectin (0.5×10^{-7} - 2×10^{-7} M),

PAI-1 promotes cell migration by competing with uPAR and releasing the cell from the Vitronectin attachment. In human fibrosarcoma HT-1080 cells, which use uPAR and integrin as anchors to Vitronectin, addition of PAI-1 itself did not detach these cells from the Vitronectin-coated surface. Sequential addition of uPA and PAI-1 promoted cell detachment, which suggests this PAI-1 function requires uPA. Binding of PAI-1 to the uPA-uPAR-integrin complex initiates internalization of the complex, which is mediated by LRP (Chazaud, Bonavaud et al. 2000). PAI-1 and uPA are then degraded in the lysosomes, but uPAR, integrin and LRP are recycled back to the cell surface. Disengagement of the adhesive receptors uPAR and integrin from the ECM promotes cell detachment. Interfering with the formation of the PAI-1-uPA-uPAR complex or the binding of this complex to LRP significantly suppresses cell motility (Chazaud, Bonavaud et al. 2000).

3-2-5-3 PAI-1 affects the signalling pathway to promote cell migration.

PAI-1 as a signalling molecule can enhance cell migration (**Figure 11**). Indeed, the different conformations of PAI-1 (active, latent, cleaved) can bind LRP1 and activates JAK/STAT1 dependent migration in chemotaxis and monolayer wound healing assays (Degryse, Neels et al. 2004), (Kamikubo, Neels et al. 2009), (Hou, Zheng et al. 2002). Addition of PAI-1 rapidly and transiently changes the cell morphology by reorganizing actin skeleton during cell migration (Degryse, Neels et al. 2004). Cell migration is inhibited by blockage of the interaction between PAI-1 and LRP through either mutation of LRP binding site on PAI-1 or LRP antagonist (Degryse, Neels et al. 2004). It is unclear whether PAI-regulated cell migration through the LRP1 receptor requires PAI-1 interaction with the ECM. Indeed, PAI-1 regulates levels of cell surface integrins by triggering their internalization in an LRP-dependent manner (Czekay, Aertgeerts et al. 2003), (Pedroja, Kang et al. 2009), (Czekay and Loskutoff 2009) resulting in cell detachment from a variety of substrates (Czekay, Aertgeerts et al. 2003), (Czekay and Loskutoff 2009). However, the mechanisms supporting PAI-1 regulated migration directly via LRP1 appears to differ from that which PAI-1 modulates cell detachment, as uPA is required for detachment, but not for the migratory response (Degryse, Neels et al. 2004), (Kamikubo, Neels et al. 2009), (Czekay, Aertgeerts et al. 2003), (Czekay and Loskutoff 2009). PAI-1 can use multiple pathways to impact on cell migration through LRP1. Ligand binding to LRP1 affects Schwann cell motility through activation of the Rho family GTPases (Mantuano, Jo et al. 2010). Notably, Rho Family GTPase activity has been connected with enhanced expression of JAK/STAT signalling (Loucks, Le et al. 2006),

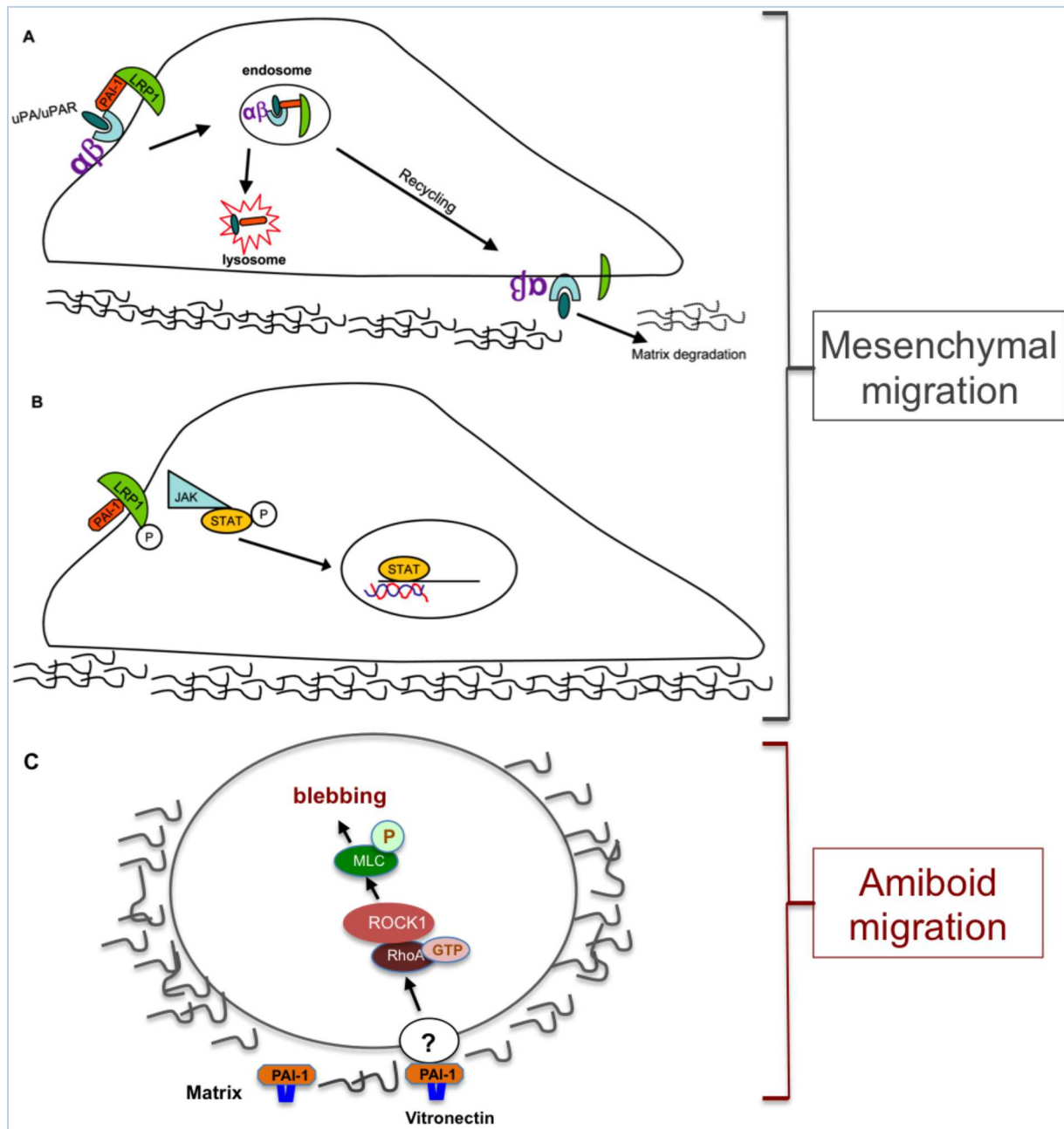


Figure 11. Involvement of the PAI-1 in cell migration.

PAI-1 contributes to wound repair by inducing mesenchymal cell migration: Two possible pathways. (A) Pathway 1: PAI-1/uPA/uPAR (uPA receptor)/LRP1/integrin complexes are endocytosed with subsequent uPAR/LRP1/integrin redistribution to the leading edge, causing an “adhesion-detachment-readhesion” cycle that promotes cell migration. (B) Pathway 2: PAI-1 binds to LRP1 in a uPA/uPAR-independent manner triggering JAK/STAT1 pathway activation resulting in stimulation of cell motility. (C) Amoeboid migration : Active, matrix-bound PAI-1 blocks the association of Integrin and uPAR with the ECM and subsequently supports cell blebbing by activating the RhoA pathway (implicating an unknown co-actor).

Adapted from (Simone, Longmate et al. 2015), (Cartier-Michaud, Malo et al. 2012).

(Pelletier, Duhamel et al. 2003). The potential contribution of these interactions in PAI-1-stimulated migration via the LRP1 remains to be determined. Consequently, even though active PAI-1 is routinely cleared from the extracellular environment in a complex with uPA-uPAR-LRP1, latent and cleaved species of PAI-1, with a preserved migratory function, remain embedded in the matrix to sustained cell migration. This paradigm supports the correlation of high PAI-1 levels with poor prognosis.

3-3 PAI-1 in regulation of angiogenesis and tumour progression

Due to the capability of uPA of mediating proteolytic degradation of ECM proteins, uPA would be expected to act anti-adhesively, while PAI-1 would be expected to promote adhesion. On the other hand the positive effect of uPA in stimulating cell migration through its ability to activate plasminogen is consistent with the concept that proteolysis would facilitate the penetration of the ECM by tumour cells. Accordingly, PAI-1 would be expected to inhibit plasminogen activation-dependent cell migration. However, reports on the activity of PAI-1 on cell migration and tumour cell invasion studied *in vitro* have been the subject of controversy. Nevertheless, consistent data from the clinical studies suggest that PAI-1 at levels found in tumour extracts is involved in promoting cancer progression. A possible mechanism by which PAI-1 promotes cancer progression is by enhancing angiogenesis. In PAI-1-deficient mice, tumour angiogenesis and progression are greatly impaired, suggesting a role of PAI-1 in new blood vessel formation (Bajou, Noel et al. 1998). Restoration of PAI-1 expression in the PAI-1 deficient mice promoted local invasion and tumour vascularization of transplanted malignant keratinocytes (Bajou, Noel et al. 1998). PAI-1 acts by promoting endothelial cells migration from Vitronectin-rich perivascular space toward fibronectin-containing tumour tissue (Isogai, Laug et al. 2001). Excessive ECM and basement membrane proteolysis hinder angiogenesis because there is the absence of a scaffold for migrating endothelial cells to adhere (Pepper 2001), (Bajou, Maillard et al. 2004). PAI-1 prevents excessive proteolysis, which therefore supports angiogenesis (Pepper 2001). PAI-1 can bind to Vitronectin, and when PAI-1 binds to uPA it loses affinity for Vitronectin (Andreasen, Egelund et al. 2000), (Sidenius and Blasi 2003). In this manner, PAI-1 could regulate cell adhesion: when PAI-1 interacts with Vitronectin it inhibits cell adhesion while when PAI-1 complexes with uPA adhesion is promoted (see section 3-2-4; (Deng, Curriden et al. 1996)). In the absence of uPA, cells would be nonadherent and motility of the cells would increase

(Deng, Curriden et al. 1996). When PAI-1 is bound to Vitronectin it can block the binding of Vitronectin to integrin $\alpha v\beta 3$, which leads to a decrease in migration on the substratum (Stefansson and Lawrence 1996). Binding of PAI-1 to uPA restores binding of Vitronectin to integrin $\alpha v\beta 3$, therefore increasing cell migration on the substratum (Stefansson and Lawrence 1996).

In summary, PAI-1 has a proangiogenic activity through its antiprotease and Vitronectin-binding function. However, the activity of PAI-1 on angiogenesis is dose-dependent. Low level (physiological level) of PAI-1 in the host may facilitate angiogenesis and tumour growth. Conversely, administration of high level (pharmacological level) of PAI-1 seems to prevent the angiogenesis, tumour growth, and metastasis (Devy, Blacher et al. 2002), (Stefansson, Petitclerc et al. 2001). For example, high levels of PAI-1 expression in human or mouse cancer cells were associated with the retardation of tumour growth, invasion, and metastasis in immunodeficient mice (Ma, Gerard et al. 1997), (Praus, Collen et al. 2002), (Soff, Sanderowitz et al. 1995). Application of high level of PAI-1 protein to immunodeficient mice bearing transplanted human tumours caused tumour growth inhibition (Jankun, Keck et al. 1997), but low level of PAI-1 enhanced tumour growth (McMahon, Petitclerc et al. 2001).

3-4 PAI-1 in regulation of cell apoptosis

A further mechanism by which PAI-1 may enhance cancer progression is by blocking apoptosis and thus enhancing cell survival. PAI-1 may modulate cell survival/death through multiple mechanisms. First, PAI-1 through its anti-proteolytic activity increases cell adhesion, which may, in turn, increase pro-survival signalling. Indeed, PAI-1 stimulates keratinocyte adhesion and rescues these cells from plasminogen-induced substratum detachment/anoikis (Providence, Higgins et al. 2008). Second, PAI-1 in complex with uPA-uPAR binds to LRP1 and induces pro-survival signalling into the cells. Third, PAI-1 mediated cell detachment/anoikis through binding of PAI-1 to Vitronectin, which prevents binding of Vitronectin to integrin $\alpha v\beta 3$. Early reports showed that addition of recombinant PAI-1 to tumour cells *in vitro* inhibited cytotoxic drug-induced apoptosis (Kwaan, Wang et al. 2000). Overexpression of active PAI-1 in low PAI-1-expressing breast cancer cell lines increased tumour cell survival upon paclitaxel treatment compared with cells expressing inactive PAI-1 (Beaulieu, Whitley et al. 2007), and mouse fibrosarcoma cells derived from PAI-1^{-/-} mice

were more sensitive to chemotherapeutic drugs-induced apoptosis than their counterparts derived from wild type PAI-1 expressing mice (Romer, Kirkebjerg Due et al. 2005). Little is known about the intracellular signalling pathways involved in PAI-1-mediated inhibition of apoptosis. Fibrosarcoma cell lines derived from PAI-1^{-/-} mice have an increased level of spontaneous apoptosis that is associated with a decreased activation of the PI3K/AKT cell survival pathway (Romer, Larsen et al. 2008). Similar to endothelial cells, PAI-1 inhibits Fas-mediated apoptosis in several human cancer cells, including brain metastasis through its control over pericellular plasmin activity (Fang, Placencio et al. 2012), (Valiente, Obenauf et al. 2014). PAI-1 also affects intrinsic apoptosis as the absence of extracellular PAI-1 in tumour cells results in higher levels of activated caspase 9 (Balsara and Ploplis 2008). Intracellular PAI-1 also promotes cell survival through its ability to inhibit caspase-3 protecting tumour cells from chemotherapy-induced apoptosis (Schneider, Chen et al. 2008).

3-5 Targeting PAI-1 in cancer

Small inhibitors of PAI-1 have been initially developed with the concept that PAI-1 inhibition could be valuable to promote plasmin-mediated fibrinolysis post acute thrombotic events. Based on the observations that PAI-1 paradoxically promotes tumour progression in several cancer types, interests in using inhibitors of PAI-1 in anti-cancer therapy have been generated. However, only a few studies have evaluated these PAI-1 inhibitory compounds in the malignant tumour context. One of the well-characterized small molecule selective inhibitor of PAI-1 (PAI-039/tiplaxtinin) was identified by high-throughput screening of compound libraries (Elokda, Abou-Gharbia et al. 2004). PAI-039 (Tiplaxtinin), that is orally active, has been tested in several preclinical models of vascular thrombosis in rats and dogs and shown to be effective in inhibiting plasma PAI-1 and promoting thrombus repermeabilization (Elokda, Abou-Gharbia et al. 2004), (Hennan, Elokda et al. 2005), (Hennan, Morgan et al. 2008). PAI-039 has also been shown to prevent the development of diet-induced obesity in a preclinical mouse model by inhibiting PAI-1 (Crandall, Quinet et al. 2006). PAI-039 has been shown to inhibit endothelial cell motility and angiogenesis in Matrigel implants in mice (Leik, Su et al. 2006). In addition, other evidences that inhibition of PAI-1 activity may be of therapeutic benefit in cancer management were provided by a study, which demonstrated the inhibition of invasiveness of human HT1080 fibrosarcoma cells and melanoma BLM cells by anti-PAI-1 antibodies (Brooks, Slomp et al. 2000).

Subsequently, several small molecule inhibitors of PAI-1 were tested for their anti-cancer activity. XR5967, a diketopiperazine that inhibits the activity of human and murine PAI-1, has been shown to dose-dependently inhibit the invasion/migration of human endothelial cells in an in vitro angiogenesis model as well as inhibit the invasion of human HT1080 fibrosarcoma cells through Matrigel (Brooks, Wang et al. 2004). Two other PAI-1 inhibitors, SK-216 and SK116, have been tested in Min mice. Min mice possess a defect in the adenomatous polyposis coli gene that provokes the formation of intestinal polyps along with serum triglyceride levels up to 10-fold higher than normal mice. They also exhibit increased serum PAI-1 levels, hepatic PAI-1 mRNA levels and stronger PAI-1 immunostaining in small intestinal epithelial cells. The administration of SK-216 and SK116 to Min mice reduced both PAI-1 activity and expression levels, and concomitantly suppressed intestinal polyp formation, indicating that PAI-1 inhibition could play a role in the prevention of colorectal tumours (Mutoh, Niho et al. 2008).

RELEVANCE AND FOCUS OF MY PROJECT

Metastasis is the primary cause of death in patients with solid tumours and patient outcomes correlate negatively with the extent of metastatic spread at diagnosis. Despite progress in the field, many questions remain and a consensus has not been reached concerning the mechanisms controlling epithelial cancer cells dissemination, a critical step in metastatic spread. The answers to these questions will lead to improvements in patient diagnostics and metastatic therapy.

As discussed in the introduction chapter, metastasis initiates with dissemination, the escape of epithelial cancer cells from the primary tumour into the surrounding stroma. The overall aim of my project was to acquire a deeper knowledge on the role of tumour microenvironment factors in tumour dissemination. It is now increasingly appreciated that progression of malignant tumours does not depend exclusively on cell-autonomous properties of the cancer cells, but is also deeply influenced by tumour stroma reactivity and undergoes a strict microenvironmental control. The crosstalk between the tumour mass and the surrounding stromal cells leads to the secretion of several molecules that increase potentially the chances for metastatic escape from the initial tumour. One model for dissemination is based on the fact that dissemination requires the loss of epithelial cell-cell junctions, where a change in the expression of intercellular adhesion genes through the EMT process could be the initiating event. Several extracellular secreted molecules were proved to promote individual cell detachment from the tumour by triggering the loss of epithelial features either by the induction of EMT by factors such as TGF-beta, SDF-1 or through the secretion of matrix remodelling proteins such as MMPs that can proceed in E-cadherin cleavage. However, cancer cells of epithelial origin frequently invade collectively in groups while retaining cell-cell junctions. The molecular regulation of this process is not well understood. We chose to study the role of PAI-1 because the plasminogen activation system formed by uPA and its inhibitor PAI-1 is a key protease cascade involved in tumour invasion. As discussed in chapter 3, PAI-1 is a biomarker of poor prognosis and plays important roles in cancer migration and invasion. Importantly, previous work had indicated that PAI-1 regulates single cell adhesion/de-

adhesion of invasive cancer cells on pro-adhesive coatings through an uPA-uPAR dependent mechanism.

In light of this, my thesis objectives were:

- 1) To elucidate adhesion abilities of non-invasive breast and colon cancer cell lines to various components of the extracellular matrix in the presence of PAI-1.
- 2) To evaluate the effect of soluble PAI-1 on cancer cell detachment from primary tumour mass employing 3D multicellular tumour spheroid assay.
- 3) To decipher the underlying mechanisms.

Part of the study was carried out on traditional 2D monolayer cultures grown on matrix-coated surface. The cell-cell or cell-matrix interactions, which are important in regulating cell invasion are lost in 2D monolayers but can be restored in matrix-based 3D systems. Therefore the study was extended to 3 dimensional Multi-Cellular Tumour Spheroid (3D-MCTS) system. In this 3D model, tumour cells are embedded into a collagen matrix as spheroids to allow cells to invade out of the tumour mass into the surrounding matrix. This model allows partial recapitulation of the tumour microenvironment and better mimics the *in-vivo* tumour mass major features (Kunz-Schughart, Freyer et al. 2004, Ivascu and Kubbies 2007, Vinci, Box et al. 2015).

RESULTS

PAPER**PAI-1 promotes tumour cell detachment in clusters leading to collective cell invasion through a ROCK and JAK dependent signalling.**

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ABSTRACT

Cancer metastasis is accepted to occur when a single cell detaches from the primary tumour following EMT to invade nearby tissues. However, there is growing evidence that cancer cells can also detach from the primary tumour as a group or cluster of cohesive epithelial cells with an extremely high metastatic potential compared to single cells. Surprisingly, the extracellular factor/s and the underlying mechanism that enable cell detachment as clusters are largely unknown. Here we show that PAI-1, a matricellular protein found in high concentration at the front of most invasive cancer microenvironment, promotes a non-EMT epithelial cancer cell detachment in cohesive clusters which move using a typical collective mode of migration characterized by a rotative movement of cells inside the cluster in breast and colon cancer cell lines. Our data support a model in which PAI-1, through the activation of LDL receptor and ROCK/JAK/STAT3/MLC2 signalling, generates actomyosin contractility and high membrane activity at the tumour periphery promoting the detachment of highly invasive cell clusters.

Highlights:

- 1- PAI-1 is promoter of collective 3D cluster formation.
- 2- PAI-1-detached clusters maintain their epithelial characteristics.
- 3- Actomyosin contractility is responsible for PAI-1 induced cluster collective invasion.

INTRODUCTION

Despite substantial efforts to improve our knowledge of cancer progression, metastasis remains the main cause of cancer death. This is due to incomplete understanding of the mechanisms by which cancer cells escape from the primary tumour to invade surrounding tissues, conducive to metastasis spread at distant secondary sites. Numerous studies have defined EMT as a pivotal process to early stage dissemination of cancer cells. EMT involves a series of sequential steps including the loss of adherens junctions, which allows cells to leave the primary tumour and the acquisition of increased motility, which drives invasion. EMT is thought to be associated with profound phenotypic changes including loss of epithelial features concomitant to acquisition of mesenchymal-like phenotype. EMT is characterized by detachment of individual cells from the primary epithelial cohesive tumour, leading to invasion of surrounding tissues, intravasation into the bloodstream and finally extravasation at distant sites. Importantly, EMT is reversible. Invasive mesenchymal-like cancer cells can reverse phenotype and become epithelial again to proliferate at metastatic sites (MET). This final reverse conversion is proposed to explain why metastasis mostly exhibits epithelial phenotypes (Gao, Vahdat et al. 2012, Lim and Thiery 2012). Because this double conversion during metastasis process is complex, the role of EMT in invasion and metastasis remains contested.

Given that there is no dramatic changes to acquire metastatic phenotype (Bernards and Weinberg, 2002) and that there is a very similar cohesive phenotype in primary epithelial cancer cells and their metastatic counterparts, it is tempting to propose that metastatic lesions initiate from groups of epithelial cancer cells sorting from the primary tumour. In accordance, EMT has been found to be dispensable for lung and prostate metastasis in *in vivo* mouse models, but induces chemoresistance in both models (Fischer, Durrans et al. 2015), (Zheng, Carstens et al. 2015). This indicates that tumour cells may not undergo EMT to form metastasis but disseminate while persisting in their epithelial phenotype (Yu, Bardia et al. 2013). These findings are in agreement with a recent study showing that multicellular groupings of primary tumour cells generate CTC clusters, which have an increased propensity to form metastasis *in vivo* (Aceto, Bardia et al. 2014). Consistently CTC clusters in patients with breast and prostate cancer correlates with poor prognosis.

However, despite the relevance of these observations, without evidence for the mechanism explaining how groups of epithelial tumour cells can leave the primary tumour, the role of non-EMT induced dissemination of cancer cells is enigmatic. In particular, the mechanism by which cancer cell clusters detach from the primary tumour and the factors involved are totally unknown.

Numerous others studies have also suggested that cancer epithelial cell invasion is not restricted to single cell migration. For many cancer types, invasion involves a group of cells, which remain cohesive (Friedl, Locker et al. 2012). In carcinoma, histopathological analysis shows that the local invasion at the tumour border mostly occurs by collective migration of grouping cells (Friedl, Locker et al. 2012). During EMT, individual cells detach from the cohesive epithelium by loss of adherens junctions. However generating collective cell migration from primary tumour raises important questions concerning the mechanism of detachment and the mode of migration. The separation of a group of cells from the primary tumour requires persistence of adherens junctions in cells comprised in the evading group, but the loss of contact with the tumour mass. Consequently collectively migrating cancer cells might adopt a specific morphological and functional organization inside the group to generate movement. Mechanisms/factors involved in such a process are still unknown.

The cell microenvironment greatly impacts on cancer cell invasion and metastasis. While several extracellular factors that promote EMT in cancers have been studied, such as TGF- β , SDF-1..., the inducers of collective cluster formation were so far poorly identified. Among the microenvironmental factors that are involved in tumour progression, PAI-1 plays a prominent role. Elevated PAI-1 is a validated marker of poor prognosis and reduced disease-free survival in breast, colorectal, lung, ovarian, gastric and oral cancers (Langenskiold, Holmdahl et al. 2009),(Duffy, McGowan et al. 2014) (Ding, Zhang et al. 2013), (Magnussen, Rikardsen et al. 2014), (Chen, Tai et al. 2014), (Nagayama, Sato et al. 1994), (Bayramoglu, Gunes et al. 2014). PAI-1 is the primary inhibitor of the plasminogen-plasmin system (PA), which modulates cell adhesion by catalyzing the degradation of the ECM (Sprengers and Kluft 1987), (Loskutoff, Sawdey et al. 1989), (Dellas and Loskutoff 2005). PAI-1 binds uPA and its receptor uPAR, the other members of the PA system. Consequently, PAI-1 decreases its affinity for Vitronectin and concomitantly increases its affinity for endocytic receptors, such as LRP (Deng, Curriden et al. 1996), (Stefansson and

Lawrence 1996),(Stefansson, Muhammad et al. 1998). PAI-1 also regulates cell migration, although the exact mechanism remains unclear. Initially described as an inhibitor of cell migration and invasion by its protease inhibitory activity (Kirchheimer, Binder et al. 1990), its potent chemoattractant activity induces cell migration (Degryse, Neels et al. 2004), (Chazaud, Ricoux et al. 2002), (Waltz, Natkin et al. 1997),(Wang, Sosne et al. 2005). Despite its activity on cell adhesion and its prominent induction upon TGF- β treatment (Freytag, Wilkins-Port et al. 2010), (Czekay, Aertgeerts et al. 2003), the role of PAI-1 in EMT or cluster formation is still obscured.

Here we show that PAI-1 induces cancer cell detachment in cohesive clusters, conducive to collective cell invasion characterized by a rotative movement resulting from a dynamic random-walk migration of cells inside the cluster. Cells constituting the PAI-1-detached clusters retain their epithelial features and E-cadherin-based cell-cell junctions. We demonstrate that PAI-1 has a de-adhesive activity which depends on LDL receptor and the activities of ROCK, JAK, STAT3 and MLC2. We evidence that, through the activation of this pathway, PAI-1 generates actomyosin contractility which promotes the detachment of highly invasive cell clusters which move using a typical collective mode of migration.

MATERIALS & METHODS

Cell culture, recombinant PAI-1 proteins and inhibitors

Human colon carcinoma cell lines, HCT116, HCT116-p21^{-/-}, HCT116-p53^{-/-} have been described (Bunz, Dutriaux et al. 1998) and were gifts from B. Vogelstein (Johns Hopkins University, Baltimore, USA). HCT116 and derivative cell lines were cultured in McCoy's 5A medium (Life Technologies) and breast cancer MCF7 cell lines were cultured in Modified Eagle's (MEM) medium at 37°C in a 5% CO₂ atmosphere. All culture medium were supplemented with 10% foetal calf serum (Fetal Calf Serum (FCS), Eurobio Abcys), 2mM L-Glutamine, 1mM sodium pyruvate, 100 U/ml penicillin and 100 µg/mL streptomycin (all from Life Technologies). HCT116 and MCF7 cells stably expressing H2B-GFP and CAAX-GFP were created by retroviral infection and cells were sorted by GFP expression to more than 95% homogeneity by fluorescence-activated cell sorting (FACS) using a FACS Aria cell sorter (BD Biosciences, Bedford, USA).

Human recombinant stable active form of PAI-1 (14-1b) (Berkenpas, Lawrence et al. 1995), referred to as PAI-1 and PAI-1 R76E mutant on 14-1b backbone referred as PAI-1 R76E were from Molecular Innovation Inc (Novi, USA). The PAI-1 R76E mutant binds to receptors in the LDL receptor family with greatly decreased affinity (Stefansson, Muhammad et al. 1998). Final concentrations of inhibitors were Y27632 (10µM; #688000), JAK inhibitor (10 µM; #420097), PD98059, (25 µM; #513001), SB203580 (10 µM; #559398), LY294002 (10 µM; #440204), Akt inhibitor (20 µM; #124017) all from Merck Millipore (Darmstadt, Germany) and, PP2 (5 µM; #P0042, Sigma, Saint Louis, USA). Control wells contained an equivalent concentration of DMSO.

Cell clustering assays

Acidified glass coverslips were coated with type I collagen extracted from rat tail tendon (40 µg/ml, #354236, BD Biosciences) or fibronectin (10µg/ml, #354008, BD Biosciences) or Bovine Serum Albumin ((BSA, control coating 80µg/ml Sigma) for 3 hours at 37° C and then washed twice with PBS. In some experiments, PAI-1 (76 µg/ml) was used as a coating protein as well to mimic matrix-bound PAI-1.

To study the effect of PAI-1 on cell adhesion, cells were trypsinized, and suspended in 0.1% serum-MEM medium (for MCF7 cells) and 0.2% serum-McCoy's 5A medium (for HCT116

cells) at a concentration of 0.25×10^6 cells/cm² in the presence or not of PAI-1 proteins and cultured for 24hrs at 37°C in a 5% CO₂ atmosphere.

To study the effect of PAI-1 on cells spread-out on monolayer, cells prepared as described above were allowed to spread for 24hrs at 37°C in a 5% CO₂ atmosphere. Then, soluble PAI-1 or PAI-1 R76E proteins or vehicle only were added at 1 µg/ml and cells were incubated for 24 h (MCF7 cells) and 48 h (HCT116 cells) at 37°C in a 5% CO₂ atmosphere.

Cells were fixed in 3.7% formaldehyde for 15 min, stained with 0.2% crystal violet and photographed using an inverted Leica DMIRB microscope using a Hamamatsu camera at x5 magnification. At least three fields were analysed per condition. Total surface occupied and cell entities (clusters and spread islands) were determined using the Cell counter plugin of the Image J software (<http://rsb.info.nih.gov/ij/>).

3D tumour spheroid invasion assay

To generate multicellular spheroid, 1000 cells per well were seeded in MEM (MCF7 cells) or McCoy's 5A (HCT116 cells) medium containing 2.4% methylcellulose in low adherence 96-well round-bottom plates. After 24 h of culture, the spheroids were collected, were embedded in a mixture (1:1) of bovine collagen solution, Type 1 (PureCol, 3.1mg/ml #5005, Advanced biomatrix, Cell Systems, Carlsbad, USA) and MEM or McCoy's 5A medium containing 0.1% FCS. PAI-1 protein (5 µg/ml) was added in the collagen mixture with or without the indicated inhibitors. The extent of tumour cell invasion is monitored at regular intervals by time-lapse microscopy over 96 h. The extent and rate of invasion achieved by single cells or by cell clusters spreading out from the tumour spheroids and invading into the matrix were quantified manually using Image J as described (Blacher, Erpicum et al. 2014, Vinci, Box et al. 2015).

Immunoblotting

Cells were harvested directly in Laemmli sample buffer and sonicated. Lysates were fractionated by SDS-PAGE and transferred to immobilon-P filters. The ECL Plus detection system with HorseRadish Peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare, UK) was used for detection.

Antibodies were as follows: anti-diphospho-MLC2-Thr18/Ser19 (#4695) and anti-Erk1/2 (137F5, #4695) both from Cell Signalling Technology; anti-P-STAT3 (Ser727) (sc-8001-R); anti-STAT3 (sc-482) and anti-phospho-Erk1/2 (E-4, sc-7383) all from Santa Cruz Biotechnology; anti-E-Cadherin (HECD-1); anti-γ-Tubulin (GTU88) and anti-MLC2 (MY-

21) both from Sigma. Bands intensities were quantified using Image J (<http://rsb.info.nih.gov/ij/>).

Immunofluorescence and microscopy

Cells on glass coverslips were fixed in 3.7% formaldehyde 15 min and permeabilized in 0.2% NP-40 for 15 min and incubated with antibodies for anti-phospho-MLC2-Ser19 (#3675, Cell Signalling Technology), E-Cadherin (HECD-1), Paxillin (#610051, BD Biosciences), ZO1 (61-7300, Invitrogen). After overnight incubation at 4°C, antibody binding was revealed with secondary Texas-Red or Alexa Fluor 488 antibodies (Molecular Probes). Fibrillar-Actin (F-actin) was stained using rhodamine-conjugated phalloidin (0.5 U/ml) (Sigma). Nuclei were counterstained with 0.2 µg/µl Hoechst. Preparations were mounted on slides using ProLong Gold (Life Technologies) Imaging of F-actin staining was carried out on an AxioPlan microscope (Zeiss) equipped with a Plan-Apochromat ×40/1.2 NA oil objective lens, an Apotom imaging system AxioVision 4.4 (Zeiss, Inc) and a cooled CCD camera (CoolSNAP HQ2, Photometrics). Z-images were analysed using Image J software. Using F-actin-staining images, cell cluster height was scored in each optical section by scrolling through the z-stack (z step of 0.5µM). Quantification of cell cluster morphology was performed using the shape descriptor 'roundness' in Image J after manually drawing around the cell cluster shape.

For the other immunostainings, images were recorded on a confocal LEICA SP5 scanning microscope. Optical sections were recorded at an interval of 500 nm, with a ×63/1.4 HCX Plan-Apochromat oil immersion lens. Excitation wavelengths were 488 nm, 561 nm, and 405 nm for the GFP-labelled- and for the Texas-Red-labelled secondary antibodies, and for the Hoechst-stained DNA, respectively.

Live-cell imaging

Multi-site light modulation contrast (LMC) and fluorescence time-lapse microscopy imaging was performed in a humidified chamber at 37°C and 5% CO₂ on a fully motorized inverted microscope (Axiovert 200M, Zeiss, Germany) using a x20/0.4 NA or a x40/0.55 Plan LMC objective lens. Images were captured with a CCD camera (MicroMax YHS 1300; Princeton Instruments, Trenton, PA) controlled by MetaMorph 7.1 software (Molecular Devices, Inc.). The exposure time was set to 50ms and images were recorded at a frequency of one image every hour (for a total recording time of 24h) or 4h (for a total recording time of 96h).

Time-lapse confocal microscopy of cells expressing H2B-GFP or CAAX-GFP was performed using a Yokogawa spinning disk CSU-XI-1 on an inverted Ti Eclipse microscope (Nikon, Japan) using x20/0.75 oil objective and an electromultiplying CCD iXon Ultra camera controlled by Andor iQ3 software. Images were treated by maximum z-projection to obtain the best intensity. Selected image sequences were assembled into movies (.avi file format) within Image J.

Intensity distribution analysis

A quantification of the peripheral intensity of expression of an immunolabelled protein relative to its central intensity of expression, in a multicellular aggregate. This quantification is carried out using 3D reconstructed images representing the whole cellular aggregate. Quantification was obtained using the Plot Profile tool and Rio-manager to manipulate of the ImageJ software. (Examples: peripheral/interior intensity ratio for Phosphorylated Myosin Light-Chain (P-MLC2) and Actin).

Statistical analysis

Mann-Whitney's tests were performed using GraphPad Prism (GraphPad Software, San Diego, USA; www.graphpad.com) was used to analyse significance between two groups. Error bars are \pm s.e.m (* P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001, ns not significant).

RESULTS

PAI-1 promotes collective cell invasion from spheroids in 3D collagen matrix

Different features of MCTS define it as a pertinent model to closely recapitulate the initial steps of invasion from a primary tumour. It is largely accepted that the MCTS is a useful *in vitro* model to mimic the *in vivo* tumour microenvironment. In particular MCTS are particularly adapted to characterize the mode of invasion of the cells into surrounding collagen matrix which is the main component of the tumour ECM (Jimenez Valencia, Wu et al. 2015). To investigate the role of PAI-1 in invasion in 3D microenvironment, colon carcinoma HCT116 cell spheroids were embedded inside 3D collagen 1 matrix.

Spheroids treatment with PAI-1 led to a marked induction of invasion into the collagen matrix. The relative invasion of tumour spheroids was determined by video-microscopy over 3 days. Interestingly, we observed dynamic extensions made of cells moving from the sphere surface inside the collagen matrix. These extensions either regressed or detached from the sphere as cell clusters, comprising 2-20 cells. Remarkably, clusters can also directly detach from the sphere into the collagen. These migrating clusters of cells leave the spheroid to enter into the collagen. Interestingly, cells within the cluster remain in contact among themselves and seemed to move collectively (Figure 1A and Supplementary videos 1 & 2 & 5). Identical results were obtained when breast carcinoma MCF-7 spheroids were treated with PAI-1 (Supplementary videos 6 & 7 and supplementary images in Figure S1).

The invasion of tumour spheroids was determined by measuring the number of extensions from the sphere at 72 hours (arrow). Number of extensions is significantly higher in PAI-1 treated spheroids, which display a 2.7 increase at 72 hours, compared to untreated spheroids (Figure 1B). We also measured the relative invasion area of spheroids over 4 days of PAI-1 treatment. Consistently, PAI-1 treated spheres showed a 1.6 fold increase of the invaded area (Figure 1C).

We next investigated how cell clusters detached from the sphere into the surrounding collagen matrix when treated with PAI-1, we expressed the histone H2B protein fused to GFP and examined the dynamics of cell detachment from the sphere. We observed a cluster formed from at least 4 cells, were detaching from the sphere treated with PAI-1 over 72 hours.

While the cluster is evading away from the sphere, it emits two pseudopodia in the direction of its movement (arrow) and importantly, cells inside this cluster displayed a rotating movement, suggesting that invasion resulted from dynamic random-walk migration of cells inside the cluster (Figure 1D and supplementary videos 3 &4). The number of cell clusters detached from the spheres was 3.9 times greater when spheroids were treated with PAI-1 (Figure 1E). The invasive ability of clusters was determined by measuring the number of clusters travelling at 300 μm from the sphere center. We observed a significant increase in the number of clusters found at 300 μm in the presence of PAI-1 compared to control suggesting that PAI-1 induced clusters migrated further away from the sphere and are more invasive (Figure 1F). In addition, the migration speed of detached clusters in presence of PAI-1 varies between 0.4-4.3 $\mu\text{m}/\text{hour}$. (Supplementary figure S2).

We classified dynamic extensions emitted from the sphere in two different types according to their shape. Extensions can be formed either by a column of adherent single-cells constituting a tube-like extension. Alternatively, extensions can also be formed by a bulk group of cells which adopt a pyramidal spatial organisation starting from the sphere edge and which have higher width and greater thickness than tube-like extensions. As observed during recording, tube-like extensions give rise to escape of individual invading cells into the matrix, whereas bulky extensions trigger the escape of cells in clusters (Figure 1G). PAI-1 significantly decreased the number of tube-like extensions at 72h and increased the number of bulky extensions (Figure 1H). Consistently, the number of individual cells detached from the sphere decreased in PAI-1 treated spheres (Figure 1I).

Altogether these results demonstrate that PAI-1 promotes cell detachment in clusters from spheroids in 3D to allow invasion into the surrounding matrix.

PAI-1 induces cluster formation through a de-adhesive effect.

To specifically assess the role of PAI-1 in supporting cell clustering, we then treated 2D- cell culture of HCT116 cells with PAI-1 at the time of seeding. Isolated HCT116 cells rapidly aggregated and organized as 3D multilayer clusters, while non-treated cells spread as monolayers (Figure 2A). We notice that clusters formed upon PAI-1 treatment were not

totally detached from the plate. Adherence to the substrate is maintained through border cells at the underside of the cluster.

Cell migration in 2D depends on cell adherence to the substrate. For example, in a low adhesive 2D environment, migration of cancer cells might be reduced because cells have weak contact to their substrate (Fackler and Grosse 2008), (Lammermann and Sixt 2009), (Friedl and Wolf 2010). Analysis of single cell trajectories before clustering indicated that directionality, velocity and distance covered by PAI-1-treated individual cells were decreased as compared to untreated cells (Figure 2C). This is in favour of a de-adhesive action of PAI-1 in 2D. In the contrary, HCT116 p21^{-/-} cells, known to be deprived of E-Cadherin cell-cell junctions, were not able to be organized in 3D multilayer clusters and stayed in monolayer, although they were more grouped in comparison with untreated cells (Figure 2A). To extend this observation to another cancer cell type, breast cancer MCF7 cells were treated with PAI-1. Again cells rapidly organized in 3D multilayer clusters (Figure 2B). This suggests that PAI-1-mediated clustering in 3D multilayers requires intercellular junctions. As cell detachment may depend on extracellular matrices, MCF7 were seeded on BSA, Collagen or Fibronectin-coated plates before PAI-1 treatment. In all conditions, cluster formation was observed, suggesting that PAI-1 effects disrupt cell adhesion, independently of the substrate (Figure 2D). To assess whether the role of PAI-1 may be to compete with the binding of cells with the substrate, MCF7 cells were seeded on immobilized, active PAI-1 coated plates. In these conditions, cluster formation was also observed, indicating that cell aggregation in multilayer clusters is supported by an active role of PAI-1 signalling, either matrix-bound or soluble (Figure 2E).

To clarify the role of PAI-1 on cell detachment, MCF7 cells were seeded 24h before PAI-1 treatment. Pre-seeding allows cells to interact with neighbouring and to be organized in flat monolayer plaques. Addition of soluble PAI-1 disrupted this organization and induced multilayer clusters (Figure 2F, 2G and Supplementary videos 8 & 9 and supplementary images in Figure S3). Pre-seeding of cells on BSA, Collagen or Fibronectin-coated plates had no effect on PAI-1-mediated cell aggregation in clusters.

Altogether our data indicate that PAI-1 has a de-adhesive effect on epithelial cancer cells and allows their reorganization in multicellular clusters harbouring multilayers spheroid-like shape.

PAI-1 stimulated cell clusters maintain intercellular junctions and display F-actin and focal adhesions rearrangements

To better identify the changes in cell morphology induced by PAI-1 in MCF7 cells cultured in 2D, we quantified different parameters of the multilayer clusters generated by PAI-1 compared to spread islands cultured on BSA coating. Total surface areas and mean surface areas occupied by cell clusters were respectively decreased by 2.9 fold and 2 fold in PAI-1 treated condition compared to non-treated condition (Figures 3A and B). PAI-1 treated clusters perimeter decreased by 4-fold compared to control (Figure 3C). The mean circularity of cell aggregates in presence of PAI-1 was significantly increased by nearly 50%, indicating a rounded form of cell clusters in PAI-1 treated condition compared to control (Figure 3D). PAI-induced-3D multilayer clusters show a significant 2-fold increase in height (33 μ M compared to 12-14 μ M in untreated cells) (Figure 3E). This confirms that cells are organized in multilayers in PAI-induced clusters.

Our results show that intercellular junctions are necessary to generate PAI-1 mediated multilayer clusters. The question arises as to how junctions are organized within the multicellular cluster. We first assessed the localisation of the E-Cadherin, which is prominent in epithelial adherens junctions. Immunofluorescence analysis showed that E-cadherin is maintained at cell-cell junctions in PAI-1-induced clusters and displays the same localisation as in untreated cells (Figure 3F). Quantification by western blotting showed no significant difference in E-cadherin expression between PAI-treated or non-treated conditions (Figure 3G). Thus, PAI-1 did not affect E-Cadherin localisation and expression. Likewise, we observed no change in ZO-1 localisation, indicating that tight junctions are not affected by PAI-1 (Figure 3J).

Paxillin plays important roles in assembly and disassembly of FAs which connect the actin cytoskeleton to the ECM. Paxillin localized at the bottom boundaries of PAI-1-induced clusters, staining the peripheral base of clusters (Figure 3K). This indicates that clusters adhere to the matrix mostly by their border cells and that PAI-1 affects Paxillin activity.

Actin cytoskeleton was organized as stress fibers in untreated cells, as expected. In PAI-1-induced clusters, actin strongly concentrated at the cluster periphery and organized as fiber bundles. In contrast, actin staining was very weak in the cluster centre (Figure 3I). This suggests that actin organization confers a contractile phenotype, which maintains cells in multilayers inside the cluster. When cells cultured on collagen were treated with PAI-1, 3D multilayer clusters were generated and actin staining displayed a peripheral localization, similar to that of PAI-1-treated cells (Figure 3I). This suggests that PAI-1 has a dominant effect on collagen. Ratios of actin fluorescence intensity at cluster periphery to cluster centre showed a significant 1.5 fold increase in PAI-1-induced clusters either seeded on BSA or seeded on collagen (Figure 3H) thus confirming the dominant effect of PAI-1 on collagen. Altogether, our results show that PAI-1 maintains intercellular junctions and reorganizes FAs and actin cytoskeleton in formed 3D multi-layered clusters.

PAI-1 de-adhesive activity depends on Low-density lipoprotein receptors (LRP/vLDLR) and the activity of ROCK and JAK kinases

Having characterized the de-adhesive function of PAI-1, we undertook to define the signalling pathway involved. PAI-1 signalling is known to be mediated through two specific pathways. The binding of PAI-1 to uPA inhibits uPA proteolytic activity and modulates uPA/uPAR-signalling activity. PAI-1 can promote cell detachment by disrupting uPAR-Vitronectin and integrin-Vitronectin interactions (Czekay, Aertgeerts et al. 2003). PAI-1 also binds to LRP and very low density lipoprotein receptor (vLDLR), and activates cell migration (Webb, Thomas et al. 2001), (Degryse, Neels et al. 2004).

To distinguish between those pathways, we treated MCF7 cells with a mutant R76E form of PAI-1, which has a dramatically reduced affinity to LDL receptors such as LRP or vLDLR but maintains its inhibitory effect on uPA. Contrary to the WT active PAI-1, the R76E mutant was unable to detach cells spread on BSA, Collagen or Fibronectin and failed to induce 3D multilayer cluster formation. This indicates that LRP and/or vLDL receptors are involved in PAI-1 effect (Figure 4A).

We then screened for signalling pathways that mediate the de-adhesive effect of PAI-1. We focused on a set of kinases largely admitted to be potent regulators of cell invasion and known to be activated by the PA system: MEK / ERK, Src, PI3K / Akt, ROCK and JAK /

STAT (Webb, Nguyen et al. 2000). Using specific inhibitors, we determined that inhibiting JAK and ROCK kinases dramatically reduced the formation of PAI-1 induced clusters, whereas the inhibition of MEK, Src and PI3K kinases had no effect (Figure 4B). When cells were seeded on collagen coated dishes, JAK and ROCK inhibitors also greatly reduced PAI-1 effects (Figure 4C). In addition, ROCK inhibition significantly increased surface area and decreased circularity in PAI-1 treated cells, supporting the notion that ROCK activity is required for PAI-1 clusters 3D structure. To strengthen the role of JAK and ROCK kinases, we evaluated their impact on PAI-1 invasive potential in 3D collagen matrix. Treatment of spheres with either JAK or ROCK inhibitors significantly decreased PAI-1-induced invasion and cluster formation in a 3D collagen matrix (Figures 4D and 4E).

These results indicate that LDL receptor/ROCK & JAK are involved in PAI-1 de-adhesive function and cluster formation.

P-MLC2 and P-STAT3 are involved in PAI-1 induced cluster formation

Clusters of tumour cells exhibit considerable flexibility to invade surrounding tissues. Our data indicate that PAI-1 induces loss of adhesion, increased invasion, actin remodelling and activation of ROCK kinase. PAI-1-invading clusters are characterized by a rotative movement of cells, which demonstrates a spectacular deformability of the multi-cellular cluster. All these indications suggest that actomyosin contractility may be activated.

Actomyosin contractility mainly results from phosphorylation of MLC2 in myosin II downstream of ROCK (Gaggioli, Hooper et al. 2007). We investigated phosphorylation of MLC2 (P-MLC2) following PAI-1 treatment. Compared to control spread MCF7 which harbour a diffused P-MLC2 staining in monolayer islands, P-MLC2 is delocalized and concentrated in cells located at the periphery of clusters induced by PAI-1 (Figure 5A). P-MLC2 is localized and concentrated at the 3D cluster periphery compared to control. Although no significant difference in the global P-MLC2 protein expression was observed in both conditions (Figure 5C), the ratio of P-MLC2 fluorescence intensity at the cluster periphery relative to its intensity at the cluster centre was significantly increased (Figure 5B). This suggests that the quantity of activated P-MLC2 is concentrated mostly at the PAI-1 induced cluster periphery. Peripheral localization of P-MLC2 suggests that PAI-1 mediated

contractility is restricted to the cluster periphery. Accordingly we conducted live imaging of 3D spheroid formed by cells transduced with CAAX-GFP construct, which label membranes and reflects their dynamic activities (Gao, Liao et al. 2009). Strikingly, CAAX-GFP labelling was restricted to peripheral small areas delineating groups of cells, which might have maintained intensive membrane activities. During recording, we noticed that spots of cells where CAAX-GFP labelling is much more pronounced preceded their detachment from the sphere thus generating invading clusters (arrow heads). (Figure 5D and Supplementary videos 10 & 11 & 12 & 13). The role of actomyosin contractility in generating tumour aggressive phenotype has been reported to be closely linked to JAK1 and ROCK kinases activities themselves positively modulating activity of the transcription factor STAT3 (Sanz-Moreno, Gaggioli et al. 2011), (Aznar, Valeron et al. 2001). To examine the implication of JAK/STAT signalling pathway, we treated MCF7 spread cells with PAI-1. Immunostaining images show a rapid translocation of P-STAT3 into the nucleus after 15 minutes of treatment, indicative of STAT3 activation (Figure 5E). Global protein expression analysis by western blotting confirmed that PAI-1 positively regulates STAT3 phospho-activation (Figure 5F). Treatment of cells with either JAK or ROCK inhibitors significantly decreases PAI-1-induced nuclear STAT3 phosphoactivation, showing that PAI-1 mediated STAT3 phosphoactivation require JAK and ROCK kinases activities (Figure 5E, 5F, 5G).

These results show that the non-homogeneous distributed increase of the contractility at the border of 3D spheroids distinguish (select) clusters of cells, which will consequently detach and invade the surrounding matrix.

Altogether our data support a model whereby PAI-1 induces actomyosin contractility in the primary tumour, which generates isolation of aggregate cells, organized in clusters of cancer cells. These clusters detach from the sphere and invade surrounding tissues using an epithelial collective mode of migration. This mode of invasion is an alternative mechanism to individual tumour cell mode of invasion generated through EMT process (Figure 5H).

DISCUSSION

Growing evidences indicate that detachment of groups of cancer cells contribute to metastasis in an EMT-independent process. Here we demonstrate that the cancer microenvironmental secreted protein PAI-1 detaches groups of cells organized in clusters which invade 3D matrices through a collective mode of migration.

Using a model of 3D-MCTS, we show that PAI-1 generates mechanic forces generated by actomyosin-driven contraction that contribute to separation of cell clusters from the sphere mass and to collective cell invasion. Extensively used as a pertinent *in vitro* model to recapitulate the *in vivo* tumour microenvironment and study the initial steps of invasion from a primary tumour, the MCTS consists of embedding multicellular spheroids inside three dimensional (3D) tumour matrices to form cell-cell and cell-matrix interactions. This model is particularly adapted to analyse the molecular and topological mechanisms of detachment and migration of cancer cells. Our results obtained in this model suggest two possible roles for PAI-1: (1) A de-adhesive role to expulse clusters of cancer cells from the spheroid embedded into a collagen matrix. (2) A promoting role in maintaining cells in clusters (cell grouping) while they evade away from the spheroid into the matrix.

Based on different morphologies, collective migration has been partitioned in different mode of migration (Friedl and Alexander 2011). Group of cells can form strands that remain epithelial structures and which protrude from the epithelial mass by forming the tip of multicellular strands. Another type of collective invasion is generated by the formation of bud-like multicellular clusters which detach from the epithelium mass and invade surrounding tissues. Our data indicate that PAI-1 can promote both types of collective migration in 3D. PAI-1-induced clusters might detach either directly from the sphere or from previously strand streaming formation (Figure 1A and Supplementary Video 1 & 2).

We also observed rotating movement of cells during the invading process. This process is similar to that observed in collective epithelial invasion during mammary branching morphogenesis which consists of multiple cells that variably changes position. (Ewald, Brenot et al. 2008). Rotating movement is described as a feature of invading groups of epithelial cells (Huang, Brangwynne et al. 2005). Coherence of the rotating movement is lost by highly transformed mesenchymal cells (Tanner, Mori et al. 2012), which usually define a leader cell

at the front of migrating cell groups to drive movement in mesenchymal collective movement (Gaggioli, Hooper et al. 2007). In our experiments, sustained rotating movement observed in PAI-1 invading clusters strengthens the conditions required for an epithelial collective mode of cancer cell invasion in 3D, lacking the use of a cell leader.

Our data indicate that E-Cadherin expression and localization are not affected by PAI-1. Increased membrane dynamic activity that precede cluster detachment from the sphere mass associated with enhanced contractility resulting from JAK/ROCK/STAT3/MLC2 signalling suggest that cluster separation is initiated by mechanical forces which isolate a group of cells in the sphere mass. Expulsion of clusters requires that these mechanical forces dominate over adherens junctions. Even we did not observe any changes in E-Cadherin localization and global expression upon PAI-1 treatment, it cannot be excluded that a transient or partial loss of E-Cadherin acts concomitantly to actomyosin contractility to facilitate detachment of cell clusters. However adherens junctions are retained inside the migrating clusters, indicating that actomyosin contractility is organized between the cells participating to the multicellular clusters. This organisation leaves E-Cadherin based adherens junction intact in the cluster, but promotes its movement. Clusters geometry and cell organization inside the cluster which move collectively in 3D environment have been described in tumour growth and metastasis. Strikingly, PAI-1 induced clusters alternatively adopt linear or tetrahedral geometries during migration (Figure 5D (arrow heads) and Supplementary videos 10 & 11 & 12 & 13), similarly to planar geometry organization defined in a computer model of cell clusters moving collectively (Vargas and Zaman 2011).

Initially described to provide contractile forces for driving rounded blebbing “amoeboid” form of single cell movement (Sanz-Moreno, Gadea et al. 2008), (Gaggioli, Hooper et al. 2007), our data provide evidence that actomyosin contractility also drive collective movement of multicellular clusters. It is striking to observe that migration of multicellular clusters share high morphological similarities with single cell rounded movement, i.e. high deformability and dependence of actomyosin contractility. In addition we observed that cells inside a cluster keep rounded shape along the migrating process. This suggests that multicellular cluster migration induced by PAI-1 adopt a rounded-collective movement.

Interestingly, it was previously reported that cells which migrate as clusters often behave in a cooperative manner where force and contractility is mediated via cell-cell junctions making the cohort behave as one big cell (Friedl and Alexander 2011), (Hidalgo-Carcedo, Hooper et al. 2011).

Consistently, our data indicate that PAI-1-induced clusters maintain cell-cell junctions (Figure 3F, 3G, 3J), whereas actin remodelling and P-MLC2 activation were distinctly localized at the cluster periphery (Figure 3I, 3H, 5A). Together, these observations suggest that PAI-1-detached clusters move as one big cell in which the increase of peripheral actomyosin contractility mimics cortical actomyosin contractility observed in amoeboid single cell migration. This is in agreement with previous findings that show a role for PAI-1 in regulating the amoeboid movement of individual cancer cells (Cartier-Michaud, Malo et al. 2012).

Actomyosin contractility generated through ROCK, JAK and STAT3 activation is required for migration of individual cancer cells in the rounded, “amoeboid” mode (Sanz-Moreno, Gaggioli et al. 2011). Silencing ROCK-driven actomyosin contractility by depletion of the RhoGAPs myosin IXA or p190RhoGAP or depletion of RhoE, lead to cell scattering and impaired collective migration of different epithelial cells (Desai, Aryal et al. 2004), (Hidalgo-Carcedo, Hooper et al. 2011), (Omelchenko and Hall 2012). With our data, those findings suggest that a fine tuning of actomyosin contractility regulate individual, but also collective migration. Different pro-inflammatory cytokines such as IL-6, LIF and oncostatin M were reported to promote single-cell invasion by inducing contractility via JAK/ROCK/STAT3 pathway (Sanz-Moreno, Gaggioli et al. 2011). However, factors that regulate the formation and invasion of cancer cell cluster were clearly lacking. Identification of PAI-1 thus provides a simple method to study collective cancer cell dissemination that would be greatly useful to better understand how invasive cells can arise from non-migrating cells.

Previous reports have shown that PAI-1 induces cell detachment through its binding to uPA/uPAR that leads to the specific inactivation of the matrix-engaged integrins (Deng, Curriden et al. 2001), (Czekay, Aertgeerts et al. 2003), (Czekay and Loskutoff 2009). PAI-1 can also induce LRP/JAK/STAT dependent migration independently of uPA activity or

Vitronectin binding (Degryse, Neels et al. 2004), (Kamikubo, Neels et al. 2009), (Hou, Zheng et al. 2002). LRP was shown to induce thyroid carcinoma cell invasion through regulating cytoskeletal organization and adhesion complex turnover (Dedieu, Langlois et al. 2008), (Langlois, Perrot et al. 2010). However all these studies are focused on the role of PAI-1 on single cell detachment and migration. Our data extend the role of PAI-1 to collective migration through LDL receptor signalling. We show that mechanical forces generated by PAI-1 induced actomyosin-driven contraction contribute to extracellular cleavage of cell clusters.

Here, we identified PAI-1 as the first described extracellular secreted protein that promotes a non-EMT epithelial cluster detachment and invasion by directly inducing actomyosin contractility through a ROCK/MLC2/JAK/STAT3 signalling pathway. PAI-1 has already been shown to promote cancer single-cell invasion. However, our work unravels a novel function for PAI-1: it can promote detachment of grouping cells and then confers invasiveness to poorly invasive epithelial cancer cells at early stages of tumour development. These findings strongly support the association between elevated PAI-1 levels and poor clinical prognosis and, thus identify PAI-1 as an appealing target for anticancer therapy. Recent evidence has shown that EMT and non-EMT processes both coexist during metastasis in controlling cancer cell dissemination and chemoresistance, respectively. Our studies indicate that PAI-1 is an ideal candidate, as it is involved both in the control of single-cell dissemination and collective tumour cell invasion. This extends the applications for anticancer agents aimed at targeting PAI-1 functions: such agents may combine the fight against chemoresistance and anti-invasive activities.

Figure 1

FIGURES

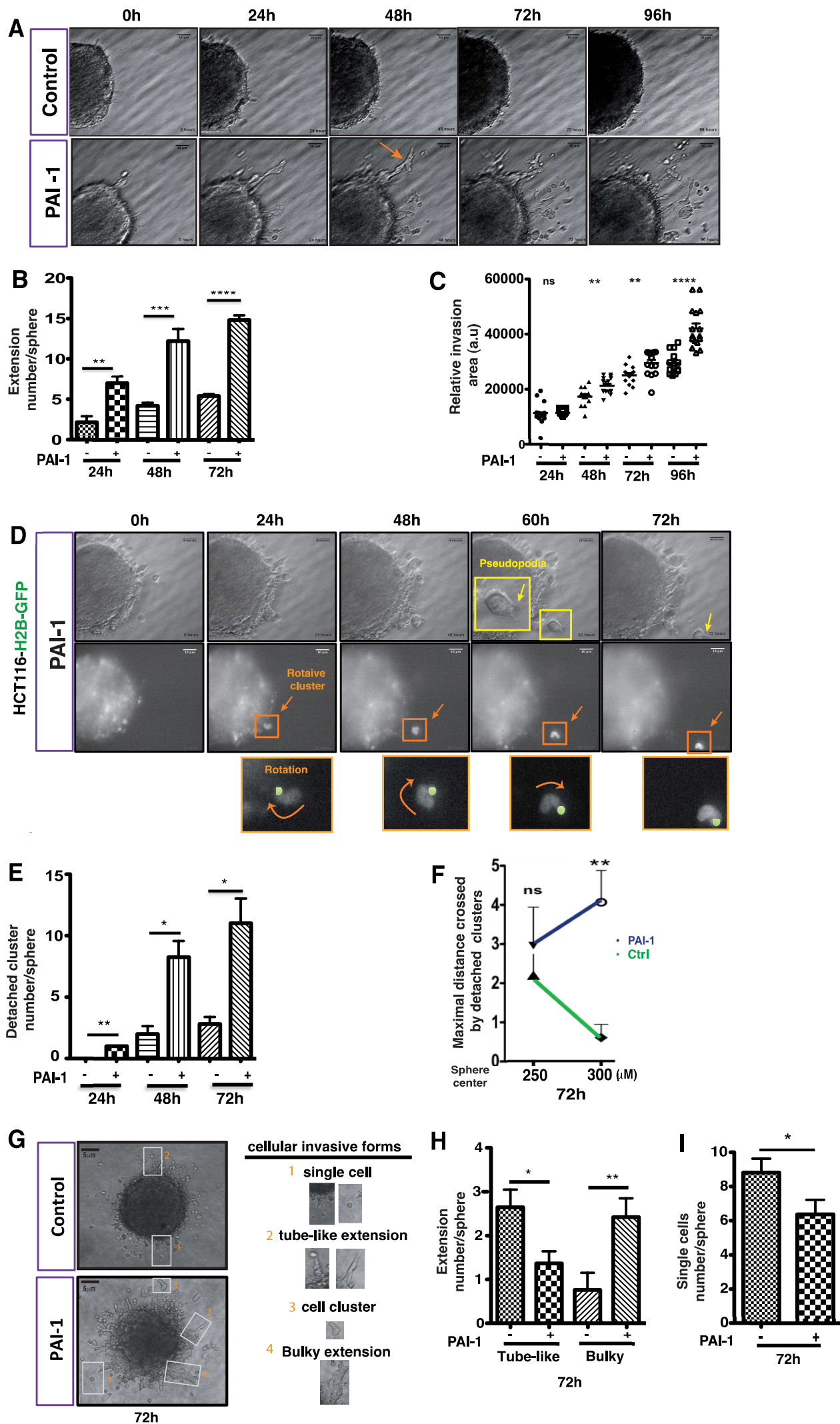


Figure 2

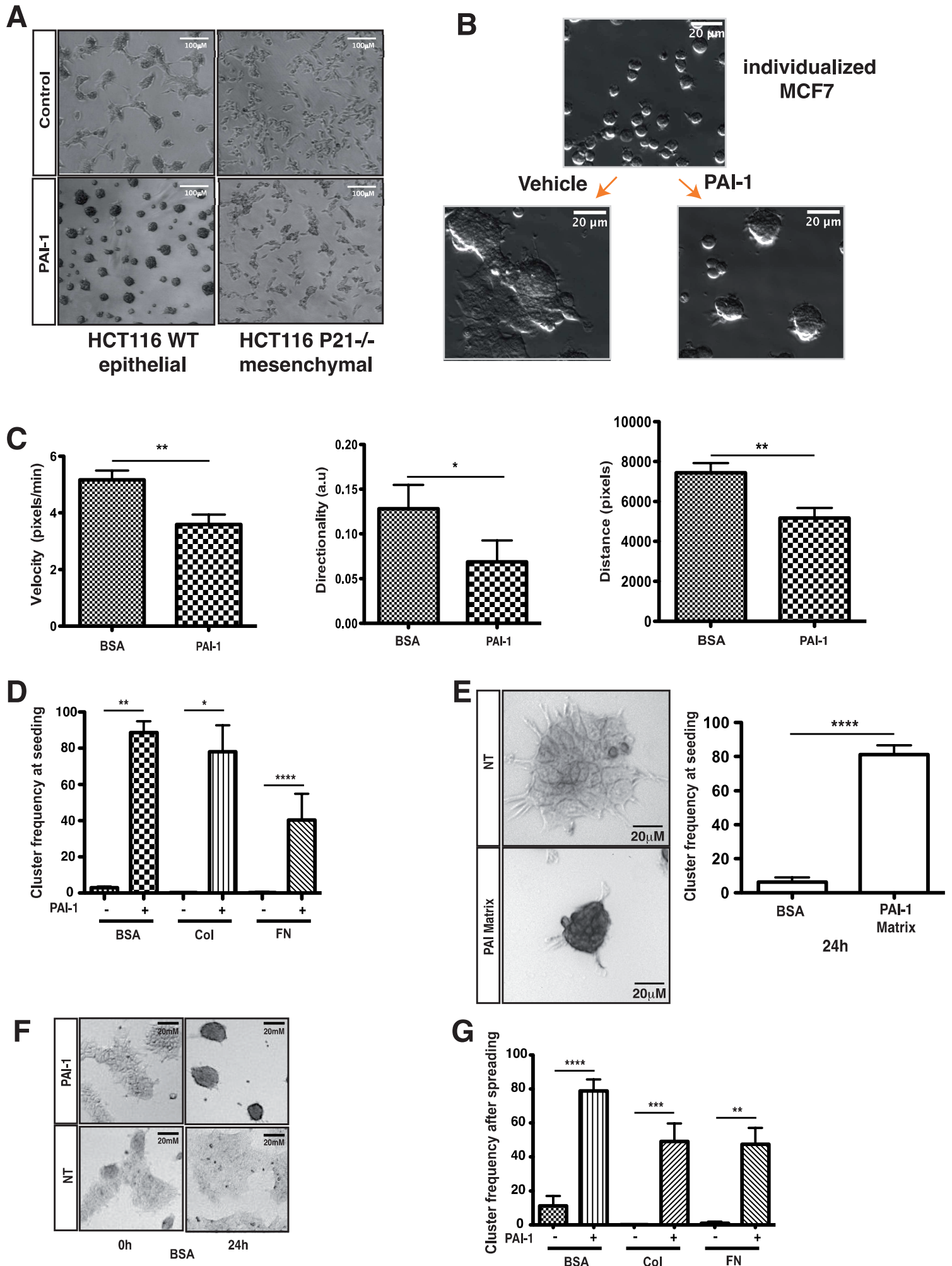


Figure 3

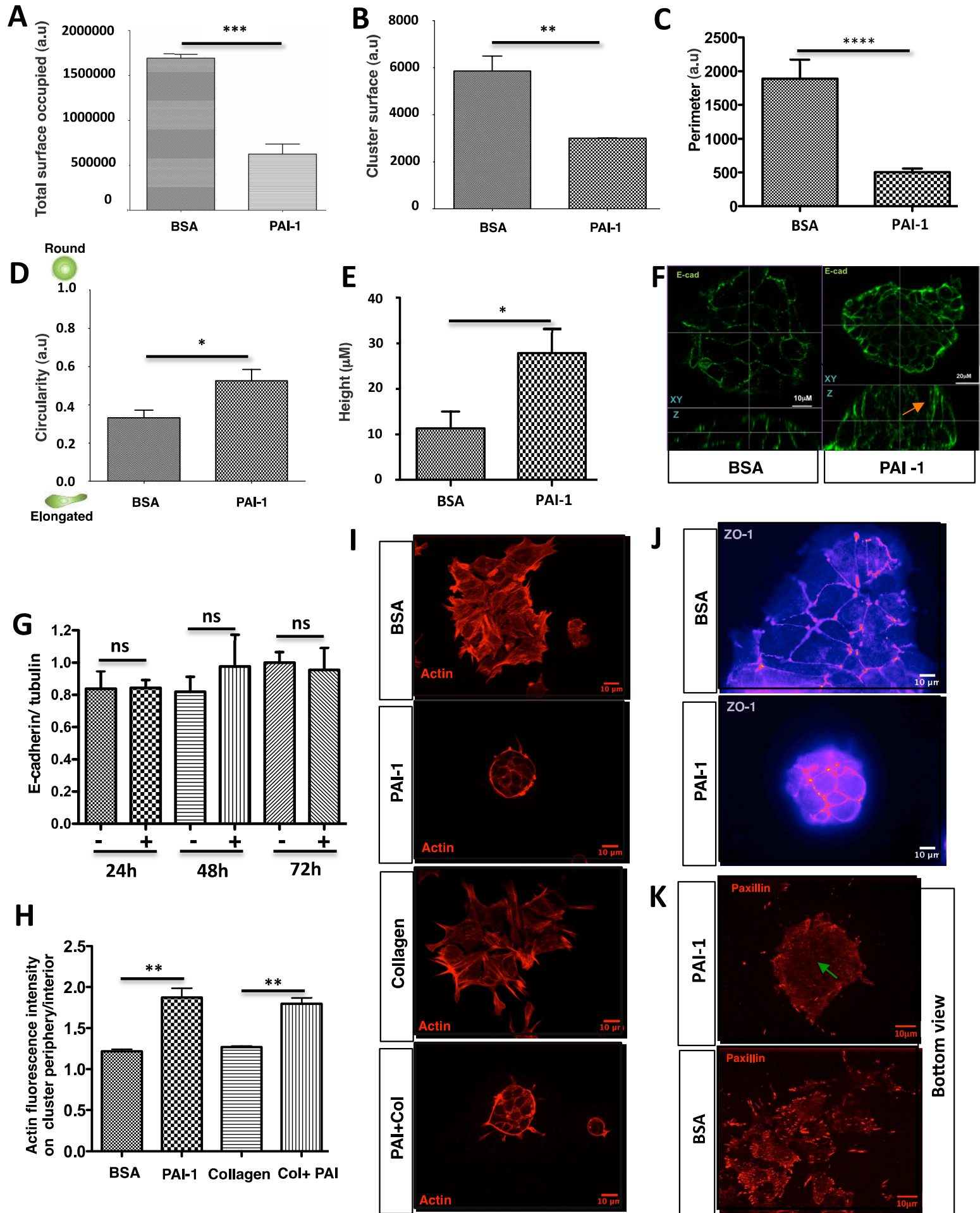


Figure 4

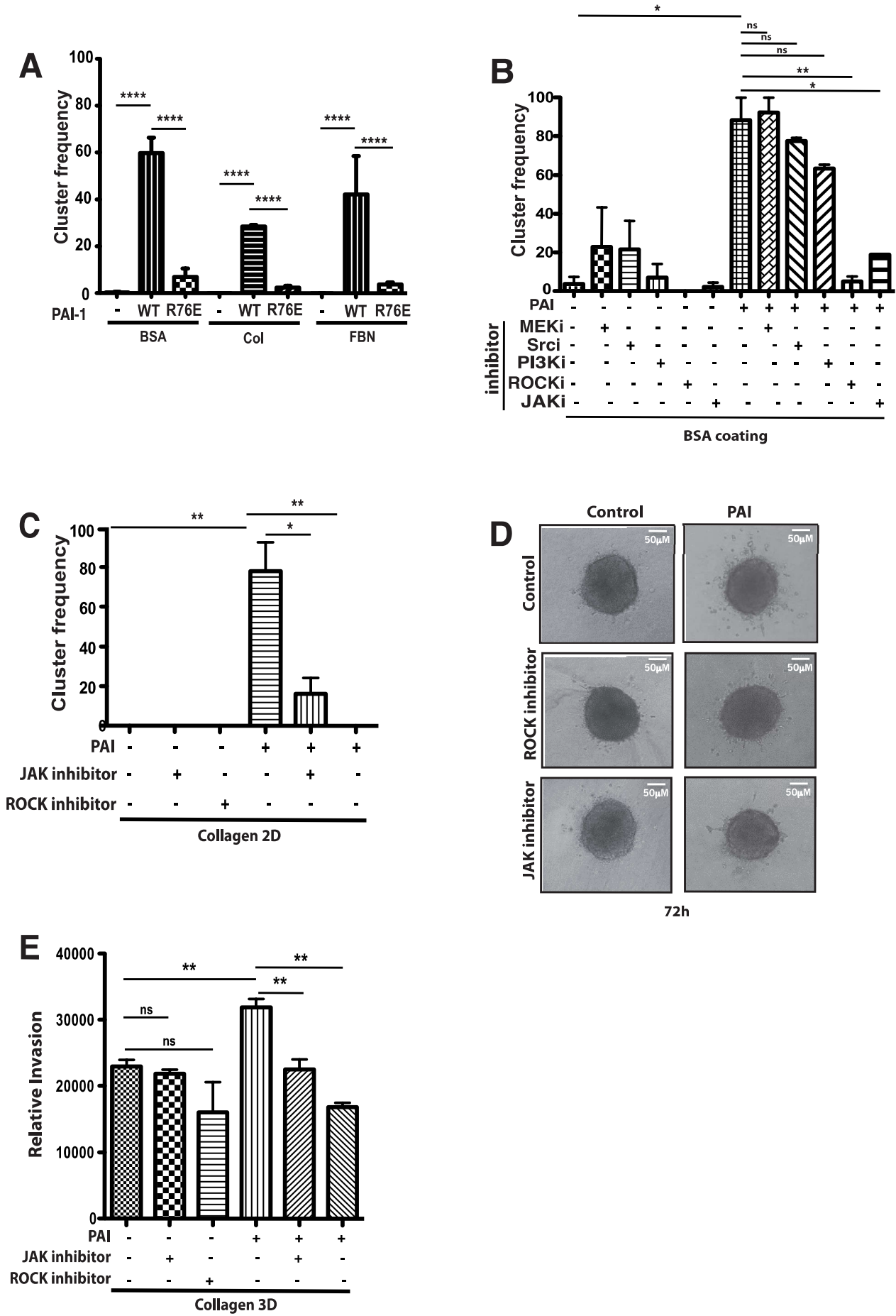
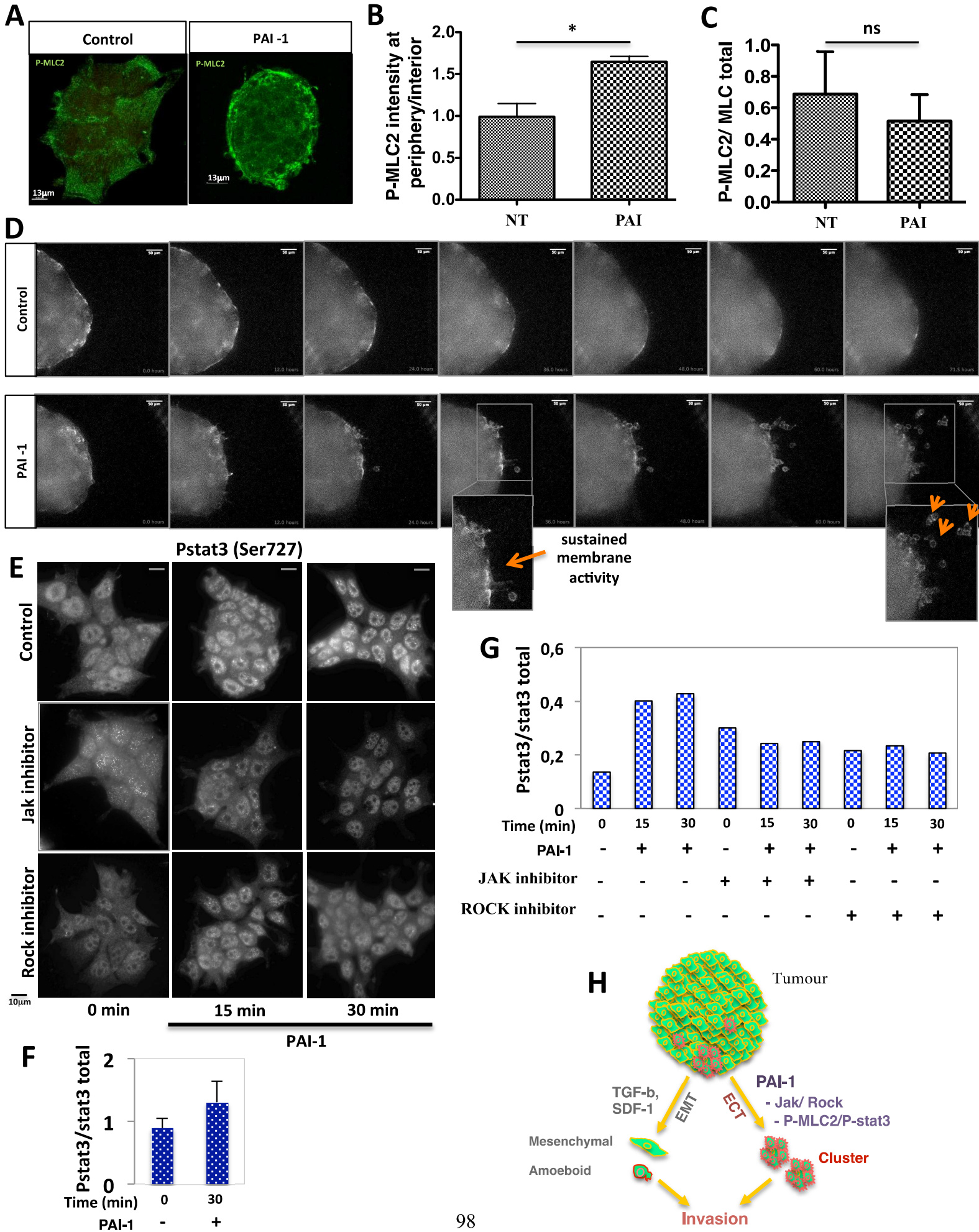


Figure 5



LEGENDS

Figure 1. PAI-1 promotes collective cell invasion from spheroids in 3D collagen matrix. (A) Phase-contrast images sequences extracted from a video-microscopy at 0h, 24h, 48h, 72h and 96h of a colon carcinoma HCT116 cell spheroid embedded inside a 3D collagen 1 matrix mixed with soluble PAI-1 14-1b. Bar, 10 μ m. (B) The number of extensions from the spheroid at 24h, 48h and 72h in presence of PAI-1 compared to non-treated spheroids was determined. Significance; ** P <0.01, *** P <0.001, **** P <0.0001. (C) The relative invasion area of spheroids at 24h, 48h, 72h and 96h for both conditions was determined. Significance; ns, ** P <0.01, *** P <0.001, **** P <0.0001. (D) Phase-contrast and GFP-fluorescent images sequences extracted from a video-microscopy at 0h, 24h, 48h, 60h and 72h of a colon carcinoma HCT116 expressing H2B-GFP spheroid embedded inside a 3D collagen 1 matrix mixed with soluble PAI-1. Bar, 10 μ m. A multicellular cluster (followed by orange curved arrows and a green point following the rotation of one cell inside the cluster) is observed detaching from the spheroid treated with PAI-1 migrated away into collagen. Two pseudopodia (followed by yellow arrows) were located at the migrating cluster front at 60h. (E) The number of detached clusters from the spheroid at 24h, 48h and 72h treated with PAI-1 compared to non-treated spheroids was determined. Data are the mean \pm s.e.m. of a representative experiment of 3 independent experiments; ** P <0.01, * P <0.05, * P <0.05. (F) The number of clusters travelled a distance of 250 μ m (proximal point) or 300 μ m (distant point) calculated from the spheroid center in PAI-1 treated (in blue) or non-treated (in green) conditions was determined. Significance; ns, ** P <0.01. (G) Phase contrast images of HCT116 spheroids after 72h of invasion with/without soluble PAI-1, showing four categories of invasive forms from spheroids : (1)single cells, (2)tube-like extensions, (3)cell clusters and (4)Bulky extensions. Bar, 5 μ m. (H) The number of tube-like and bulky extensions emitted from the spheroids at 72h for PAI-1 treated and non-treated conditions was determined. Significance; * P <0.05, ** P <0.01. (I) The number of single cells detached from the spheroids at 72h for PAI-1 treated and non-treated conditions was determined. Significance ; * P <0.05. For all quantifications unless mentioned, data are the mean \pm s.e.m. of 3 independent experiments, soluble PAI-1 14-1b concentration was 5 μ g/ml and at least 5 spheroids were considered per time point per condition.

Figure 2. PAI-1 induces cluster formation through a de-adhesive effect. (A) Phase-contrast images of a colon carcinoma Wildtype or P21-/- HCT116 individualized cells seeded on BSA control coating and treated or not at seeding time with soluble PAI-1 14-1b during 48h. Bar, 100 μ m. (B) Phase-contrast images of a breast carcinoma MCF-7 cells seeded and treated or not with soluble PAI-1 on BSA coating at 24h. Bar, 20 μ m. (C) Velocity (pixels/min), directionality (a.u) and distance (pixels) travelled by individual MCF-7 cells when seeded on BSA or PAI-1 coatings. (D) The frequency of clusters aggregated from individualized MCF-7 cells seeded on collagen, fibronectin or BSA coatings and treated or not at seeding time with soluble PAI-1 during 24h was determined. Data are the mean \pm s.e.m. of 3 to 5 independent experiments; ** P <0.01, * P <0.05, **** P <0.0001. (E) Phase-contrast images of MCF-7 cells seeded on PAI-1 or BSA coating during 24h. Bar, 20 μ m. The frequency of clusters aggregated in both conditions was determined. Data are the mean \pm s.e.m. of 5 independent experiments; **** P <0.0001. (F) Phase-contrast images of spread islands of MCF-7 cells treated or not with soluble PAI-1 14-1b during 24h on BSA control coating. Bar, 20 μ m. (G) The frequency of clusters aggregated from spread MCF-7 islands

seeded on collagen, fibronectin and BSA coatings after 24h of PAI-1 treatment. Data are the mean \pm s.e.m. of 4 to 6 independent experiments; **** P <0.0001, *** P <0.001, ** P <0.01. For all the previous quantifications, PAI-1 14-1b concentrations was at 1 μ g/ml (soluble) and 76 μ g/ml (coating). At least 10 images covering 2 wells of 1.9cm² surface per condition were considered.

Figure 3. PAI-1 stimulated cell clusters maintain their intercellular junctions and display F-actin and FAs rearrangements. (A) Total surface occupied by cell clusters, (B) the surface occupied by each cell cluster, (C) the perimeter, (D) the circularity and (E) the height of cell clusters aggregated from individualized MCF-7 cells seeded on PAI-1 or BSA coating during 24h. Significance: *** P <0.001, ** P <0.01, **** P <0.0001, * P <0.05, * P <0.05, respectively. At least 10 images covering 2 wells of 1.9cm² surface per condition were considered. (F) Localization analysis by immunostaining of E-cadherin protein in optical sections at xyz and xzy plans of cell clusters aggregated from individualized MCF-7 cells seeded on PAI-1 or BSA coating during 24h. Bar, 10 and 20 μ m. (G) Quantification following immunoblotting of E-cadherin protein relative to γ -tubulin in cell clusters aggregated from individualized MCF-7 cells seeded on PAI-1 or BSA coating during 24h, 48h and 72h. Significance: ns, ns, ns. (H) Quantification following immunostaining of F-actin protein using peripheral relative to interior intensity signals obtained from 3D reconstructions of cell clusters aggregated from individualized MCF-7 cells on BSA or collagen coatings and treated with/out soluble PAI-1 during 24h. Data are the mean \pm s.e.m. of a representative experiment for 3 independent experiments; ** P <0.01, ** P <0.01. At least four 3D reconstructions are considered for quantification per condition. (I) Localization analysis by immunostaining of F-Actin protein in a xyz optical plan of cell clusters aggregated from individualized MCF-7 cells seeded on BSA or collagen coating and treated or not with soluble PAI-1 during 24h. Bar, 10 μ m. Localization analysis by immunostaining of ZO-1 protein (panel J) and of paxillin protein (panel K) in cell clusters aggregated from individualized MCF-7 cells seeded on BSA, PAI-1 coatings during 24h (a bottom view for panel K). Bar, 10 μ m. Unless mentioned, data are the mean \pm s.e.m. of 3 independent experiments for each quantification. PAI-1 14-1b concentrations was at 1 μ g/ml (soluble) and 76 μ g/ml (coating).

Figure 4. PAI-1 de-adhesive activity depends on Low Density Lipoprotein Receptors (LRP/vLDLr) and the activity of ROCK and JAK kinases. (A) The frequency of clusters aggregated from spread MCF-7 islands seeded on collagen, fibronectin and BSA (ctrl) coatings non treated or treated during 24h with soluble active or mutant PAI-1 (R76E; no binding to LRP); **** P < 0.0001 for all comparisons. (B) The frequency of clusters aggregated from MCF-7 cells seeded on BSA coating non treated, treated with soluble PAI-1 alone or in combination with: 25 μ g/ml Of MEK inhibitor (PD98059), 5 μ g/ml of Src inhibitor (PP2), 10 μ g/ml of PI3K inhibitor (LY294002), 10 μ g/ml of ROCK inhibitor (Y27632) and 10 μ g/ml of JAK inhibitor (JAKi) during 24h. Significance; * P <0.05, ns, ns, ns, ** P <0.01, * P <0.05 (C) The frequency of clusters aggregated from MCF-7 cells seeded on collagen coating non-treated or treated with soluble PAI-1 14-1b in combination with 10 μ g/ml of ROCK inhibitor; Y27632 or 10 μ g/ml of JAK inhibitor; JAKi during 24h. Significance; ns, ns, **: P , 0.0059, * or *, P , 0.0207, **: P , 0.0059, respectively. For the previous quantifications A, B and C, Data are the mean \pm s.e.m. of 2, 2 and 3 independent

experiments, respectively and at least 10 images covering 2 wells of 1.9cm² surface per condition were considered. (D) Phase-contrast images of colon carcinoma HCT116 cell spheroid in 3D collagen matrix non-treated or treated with 5 µg/ml soluble PAI-1 in combination with 10µg/ml of ROCK inhibitor or 10µg/ml of JAK inhibitor during 72h. Bar, 50µm. (E) Quantification of relative invasion area of spheroids at 72h for conditions in (4C) was determined. Data are the mean ± s.e.m. of 3 independent experiments; ns, ns, ***P*<0.01, **P*<0.05, ***P*<0.01, respectively and at least 5 spheres for each condition were considered. Unless mentioned, concentration of soluble PAI-1 (1µg/ml) and PAI-1 coating (76µg/ml).

Figure 5. P-MLC2 and P-STAT3 are involved in PAI-1 induced cluster formation. (A) Localization analysis by immunostaining of P-MLC2 protein in easy 3D intensity reconstruction representation of cell multi-layered or spread clusters aggregated from MCF-7 cells seeded on PAI-1 or BSA coating during 24h. Bar, 13 µm. (B) Quantification following immunostaining of P-MLC2 protein in identical conditions as Fig.5A. Data are the mean ± s.e.m. of a representative experiment for 3 independent experiments; **P*<0.05. At least four 3D reconstructions are considered for quantification per condition. (C) Quantification following immunoblotting of P-MLC2 protein relative to total MLC in identical conditions as Fig.5A. Data are the mean ± s.e.m. of 3 independent experiments; ns. (D) GFP-fluorescent images sequences extracted from a video-microscopy at 0h, 12h, 24h, 36h, 48h, 60h and 71.5h of a colon carcinoma HCT116 expressing CAAX-GFP spheroid embedded inside a 3D collagen matrix mixed with 5µg/ml soluble PAI-1. Arrows show the peripheral staining at the spheroid level and arrow-heads show a cluster with marked intensity that detaches from the PAI-treated-spheroid. Bar, 50µm. (E) Localization analysis by immunostaining of P-STAT3 protein in MCF-7 spread islands on BSA coating, treated or not with 1µg/ml of soluble PAI-1 alone or in combination with 10µg/ml of ROCK inhibitor or with 10µg/ml of JAK inhibitor during 15 or 30 minutes. Bar, 10 µm. Quantification following immunoblotting of P-STAT3 protein relative to total STAT3 protein in presence of PAI-1 alone (F) or identical conditions as panel D (G). Data are representatives of one experiment. (H) Model: PAI-1 promotes cell detachment in clusters in ROCK and JAK dependent mechanism.

SUPPLEMENTARY FIGURES

Figure S1

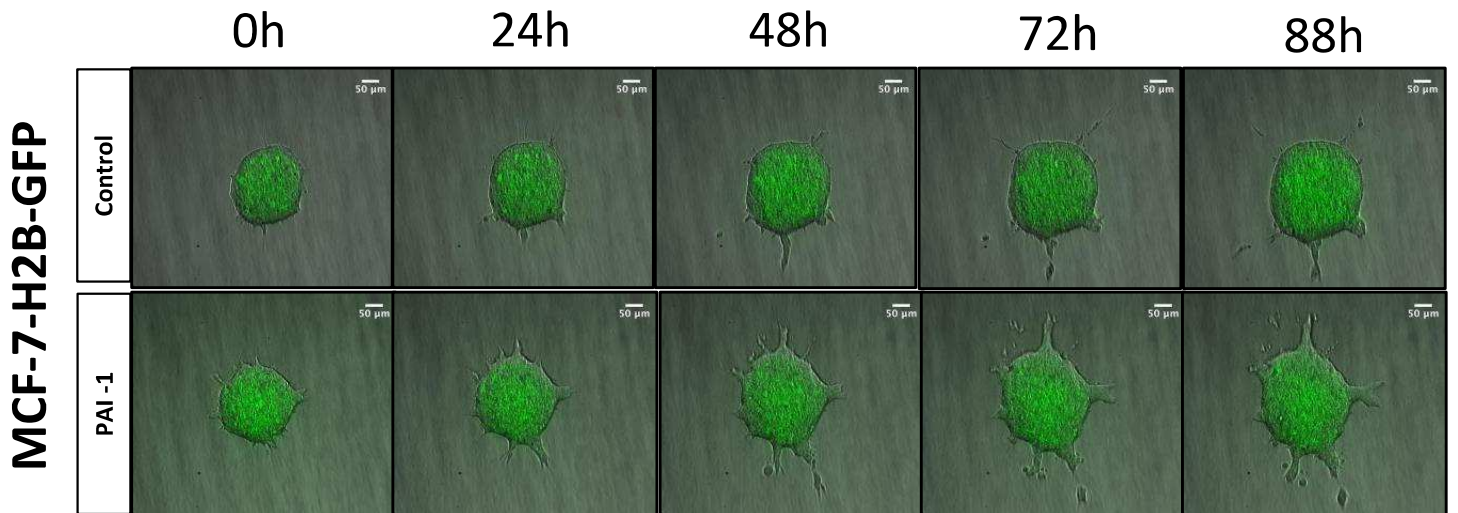


Figure S1. PAI-1 promotes collective cell invasion from spheroids in 3D collagen matrix

Merged Transmission/GFP-fluorescence images sequences extracted from a video-microscopy at 0h, 24h, 48h, 72h and 88h of breast carcinoma MCF-7 cell spheroid in 3D collagen matrix mixed or not with soluble PAI-1. Bar, 10μm. Soluble PAI-1 concentration was 5μg/ml.

Figure S2

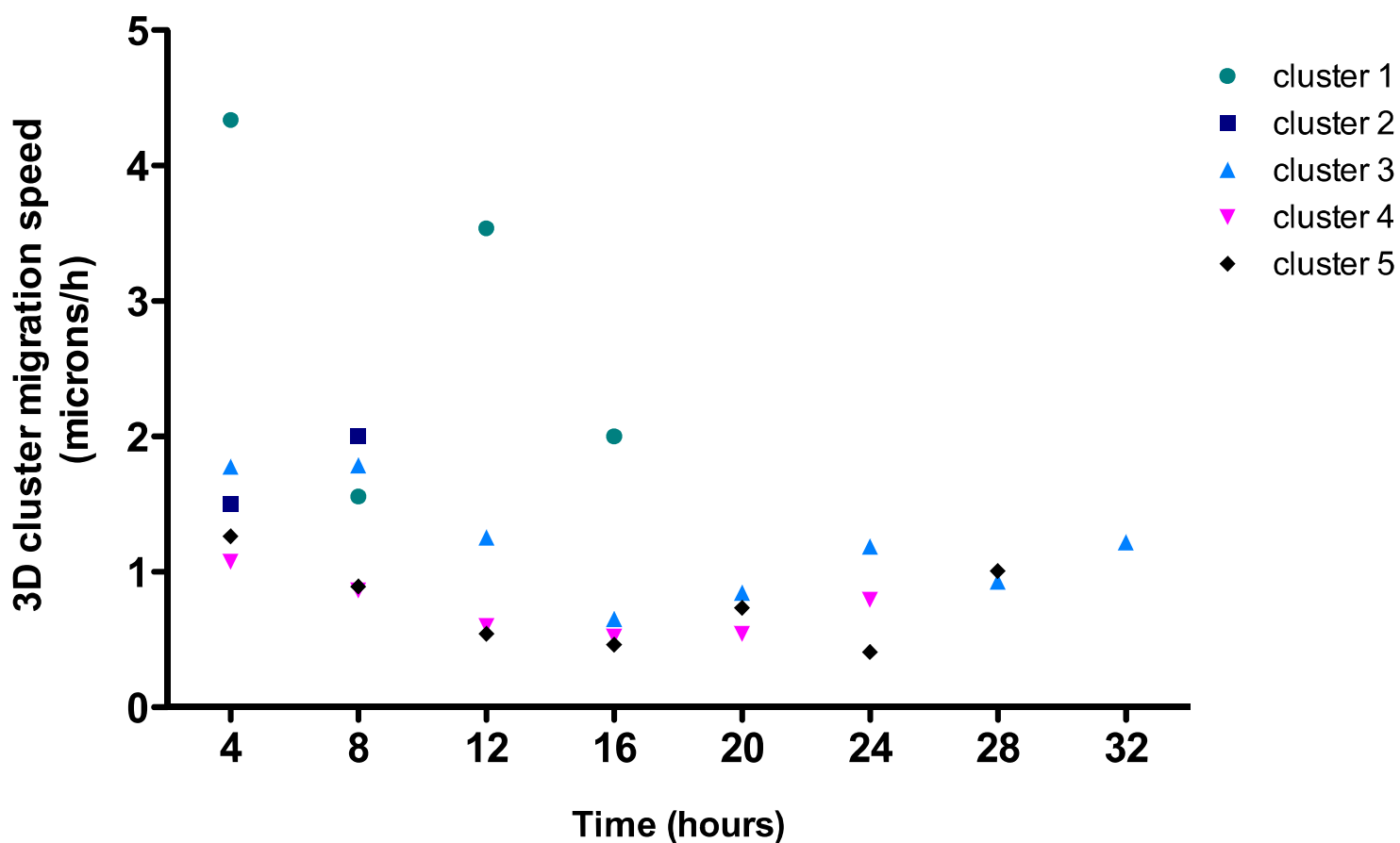


Figure S2. PAI-1 detached clusters migration speed in 3D collagen matrix

3D migration speed of detached clusters calculated each 4h for a duration of 32h from colon carcinoma spheroids embedded in collagen matrix treated with PAI-1. 5 clusters were tracked. Speed is represented in microns/hours and varies between 0,4-4,4 microns/h.

Figure S3

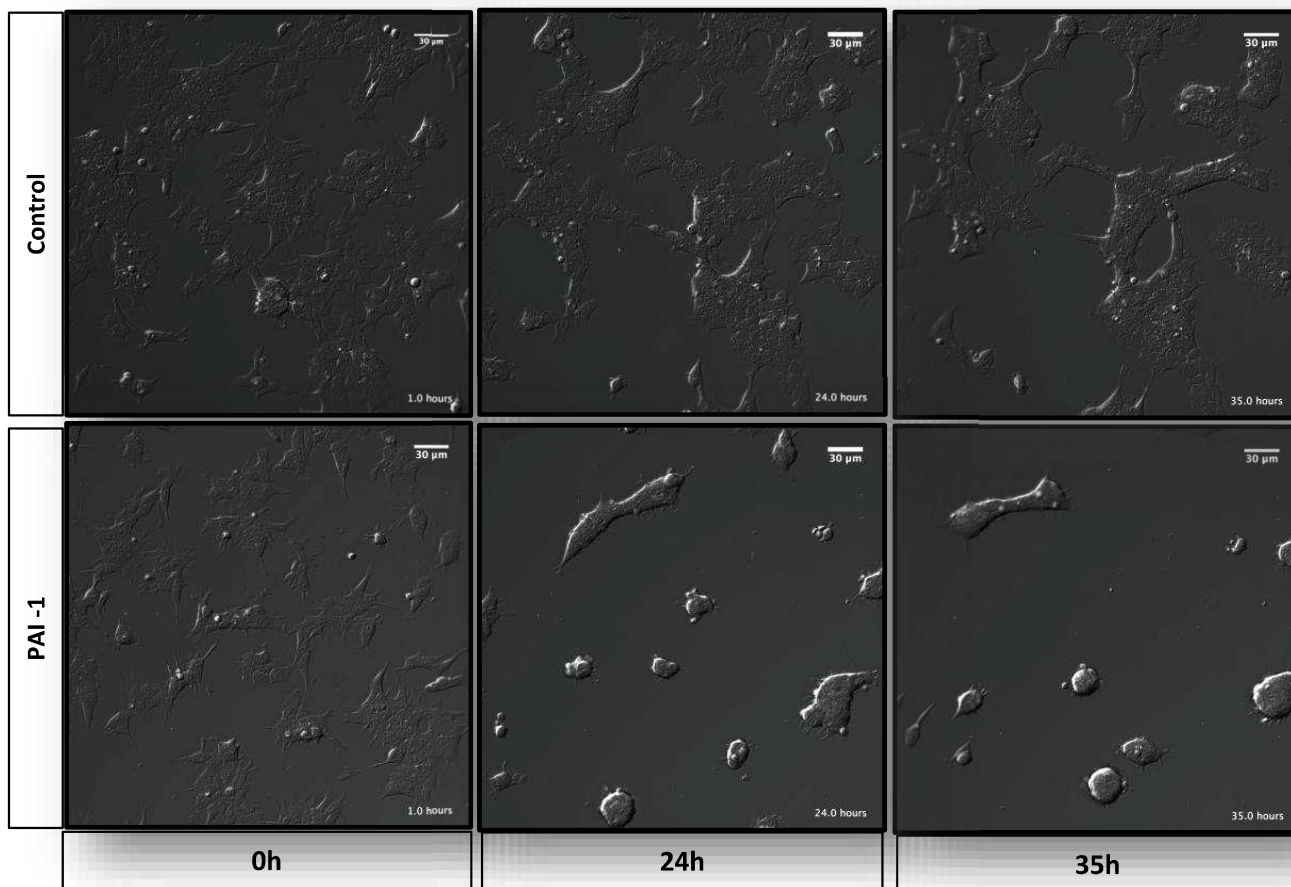


Figure S3. PAI-1 induces cell detachment to form multilayered clusters

Phase-contrast images extracted from videos sequences at 0h, 24h and 35h of spread islands of MCF-7 cells seeded on collagen treated or not with soluble PAI-1. Bar, 30μm.

Figure S4

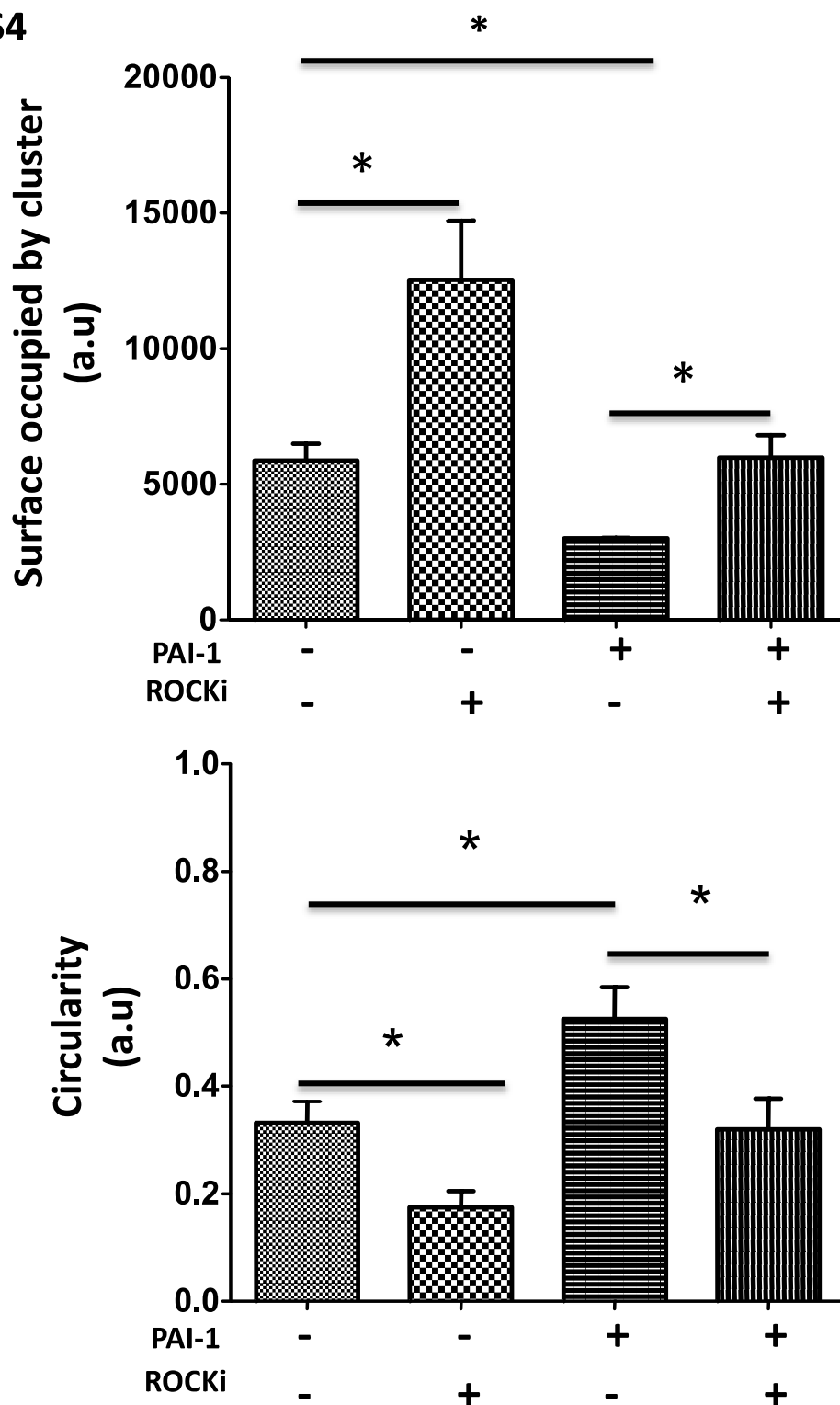


Figure S4. ROCK inhibition increases surface area and decreases the circularity of PAI-1 induced clusters

Quantification of (A) the surface occupied (B) the circularity of cell aggregates from individualized MCF-7 cells seeded on PAI-1 or BSA coating treated or not with ROCK inhibitor during 24h. Data are the mean \pm s.e.m. of 3 independent experiments for each quantification; significance for all comparisons was $*P < 0.05$. PAI-1 14-1b concentration used for coating was $76 \mu\text{g/ml}$ and at least 10 images covering 2 wells of 1.9cm^2 surface per condition were considered.

SUPPLEMENTARY MOVIES

Videos (1-2-3-4-5). PAI-1 promotes collective cell invasion from spheroids in 3D collagen matrix

- Transmission or GFP-fluorescence video-microscopy sequences of colon carcinoma HCT116 cell spheroids in 3D collagen matrix mixed or not with soluble PAI-1, 5 μ g/ml .Bar, 10 μ m.

Video 1: Non-treated, Transmission: poor or no invasion

Video 2: PAI1-treated, Transmission: cluster invasion

Video 3: PAI1-treated, Transmission: cluster rotation

Video 4: PAI1-treated, GFP-fluorescence: cluster rotation

Video 5: PAI1-treated, Transmission: cluster expulsion

- Merged transmission/GFP-fluorescence video-microscopy sequences of breast carcinoma MCF-7 cell spheroids in 3D collagen matrix mixed or not with soluble PAI-1, 5 μ g/ml .Bar, 50 μ m.

Video 6: Non-treated, merged: poor or no invasion

Video 7: PAI1-treated, merged: cluster invasion

Videos (8-9). PAI-1 induces cell detachment to form multilayered clusters

- Phase-contrast video sequences of spread islands of MCF-7 cells seeded on collagen treated or not with soluble PAI-1. Bar, 30 μ m.

Video 8: Collagen non-treated, Transmission: no detachment

Video 9: Collagen + PAI1-treated, Transmission: detachment and clusters formation

Videos (10-11-12-13). High membrane activity and dynamic movement at detachment spots on the spheroid surface

- GFP-fluorescent or phase-contrast video recordings following a video-microscopy during 71.5h of colon carcinoma HCT116 expressing CAAX-GFP spheroids embedded inside a 3D collagen matrix mixed or not with soluble PAI-1. Soluble PAI-1 concentration was 5µg/ml. Bar, 50µm.

Video 10: Non-treated, Transmission: no invasion

Video 11: Non-treated, GFP-fluorescence: no invasion

Video 12: PAI1-treated, Transmission: cluster invasion

Video 13: PAI1-treated, GFP-fluorescence: cluster invasion

NOTE: videos are available in dropbox link

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**DISCUSSION
&
PERSPECTIVES**

DISCUSSION & PERSPECTIVES

Several recent studies are increasingly supporting the fact that cell detachment of groups of cancer cells maintaining their epithelial features is largely contributing to metastasis occurrence in an EMT-independent process (Fischer, Durrans et al. 2015), (Zheng, Carstens et al. 2015). Here we demonstrate that the tumour microenvironmental secreted protein PAI-1 detaches groups of cells organized in clusters to invade 3D matrices. These results were obtained thanks to a pertinent *in vitro* model to recapitulate the *in vivo* tumour microenvironment and to study the initial steps of invasion from a primary tumour, the 3D-MCTS system. Our results obtained using a 2D coating system and extended to the 3D invasion model, suggested two possible roles for PAI-1 that we will discuss here: (1) A de-adhesive role to expulse clusters of cancer cells from the spheroid embedded into a collagen matrix. (2) A promoting role for PAI-1 in maintaining cells in clusters (cell grouping) while they evade away from the spheroid into the matrix. Using both models, we evidence that PAI-1 is involved in mechanical forces generation driven by actomyosin contractility that contribute to separation of cell clusters from the sphere mass.

PAI-1 promotes collective cluster invasion in 3D spheroid model

The MCTS of non-invasive epithelial cancer cells were embedded in the presence of PAI-1 in a collagen I matrix, a major component found in the ECM. Thereby, spheroids treatment with PAI-1 induced extensions with high dynamicity as they can either regress or detach from the sphere as cell clusters and clusters can also directly detach from the sphere into the collagen. Migrating clusters of cells leave the spheroid to evade into the collagen (Figure 1D, Supplementary videos 1, 2, 5). Interestingly, cells within the cluster remain in contact among themselves, indicative of cell-cell junctions maintenance and collective movement (Figure 3F, 3G, 3J).

Our finding is in agreement with a recent study demonstrating that CTC clusters detached from a tumour xenograft were highly metastatic compared to single cancer cells in *in-vivo* mouse model. Importantly, those clusters were detached originally from groups of cells closely situated at the initial xenograft tumour, not formed by aggregation events during their metastatic journey (Aceto, Bardia et al. 2014). Importantly, EMT has been found to be dispensable for lung and prostate metastasis in *in-vivo* mouse models, but induces chemo-

resistance in both models (Fischer, Durrans et al. 2015). This indicates that tumour cells may not undergo EMT to form metastasis but disseminate while persisting in their epithelial phenotype (Yu, Bardia et al. 2013). In coherence with those phenomena, a very similar phenotype was observed in a clinical tumour specimen where large groups or strands of cells extend and either stay connected to the tumour mass or can detach as small clusters that penetrated the surrounding tissue. It is described as the most invasive pattern of carcinoma (Friedl, Locker et al. 2012). Our results demonstrate that PAI-1 induced significant increases in the number of extensions, number of detached clusters, mostly found at far distances from the spheroid according to measurements of distance and invaded area surrounding the spheroid and more interestingly the clusters have increased migration speed inside the collagen matrix (Figure 1, Supp. Figure S2). These data suggest that PAI-1 treated spheroids were highly invasive.

As shown previously, PAI-1 plays a regulatory role in cell migration either by balancing cell adhesion and detachment activities (Deng, Curriden et al. 1996) or by affecting cell signalling (Degryse, Neels et al. 2004). It regulates directional mesenchymal cell movements or amoeboid-like cell blebbing of invasive cells based on the following observations: Addition of active PAI-1 in solution promotes directional migration of both wild type and PAI-1 deficient (PAI-1, -/-) keratinocytes (Providence, Higgins et al. 2008). Moreover, PAI-1 coating showed a capacity to maintain the blebbing of invasive colorectal carcinoma SW620 in a RhoA/ROCK dependent manner (Cartier-Michaud, Malo et al. 2012). PAI-1 was also shown to have a pro-migratory effect on highly invasive cancer cells (Chazaud, Ricoux et al. 2002). Those reports insisted on a pro-migratory role of PAI-1 mostly on single invasive cancer cells. Meanwhile, our analysis of cell trajectories in 2D model, we observed that non-invasive MCF-7 cells seeded on PAI-1 coating showed reduced velocity, directionality and traversed a shorter distance compared to MCF-7 coated on BSA coating (Figure 2C) suggesting that PAI-1 does not affect invasive and non-invasive cells in the same manner. These results support the differential role of PAI-1 towards cells expressing high or very low level of uPA described previously (Chazaud, Ricoux et al. 2002). Indeed, PAI-1 seems to promote a random cell movement of non-invasive cancer cells in 2D. It could also indicate a limitation of a 2D coating system to study non-chemotactic induced migration of non-invasive cells, as very interestingly in a 3D model, we are first to show that PAI-1 promotes collective epithelial migration of non-invasive cancer cells.

Following our detailed analysis of the qualitative and quantitative features of invasive structures in our experiments, we observed four types of invading entities originating from the spheroids: tube-like extensions and Bulky (thick) extensions, clusters or single cells. We also observed during invasion induction, that single cells originated mainly from tube-like extensions and that clusters came from bulky extensions (Supplementary videos 1 and 2). Furthermore, PAI-1 treated spheroids induced more bulky extensions, less tube-like extensions, more clusters and less single cells than in non-treated spheroids (Figure 1E, 1G, 1H, 1I). Altogether this suggests that PAI-1 promotes preferentially collective cluster detachment and invasion.

Detached clusters observed in the presence of PAI-1 seems to share a similar structure to clusters observed in clinical tumour specimen (Friedl, Locker et al. 2012). Strikingly, PAI-1 clusters in the 3D model displayed a rotating movement, suggesting that invasion resulted from dynamic random-walk migration of cells inside the cluster, without necessarily having a leader cell (Figure 1D, Supplementary videos 3 and 4) and as observed previously (Marmaras, Berge et al. 2010), (Marmaras, Berge et al. 2010), (Tanner, Mori et al. 2012), (Wang, Lacoche et al. 2013). The rotational movement of cells in a cluster is considered as a typical behavior of collectively migrating cells (Chen, Dodd et al. 2013). In addition, rotating cluster cells seem to participate in the reorganisation of ECM components and their assembly (Wang, Lacoche et al. 2013) thus suggesting a similarity with (CAFs) action to reorganize ECM molecules resulting in facilitated invasion. Rotating movement is described as a feature of invading groups of epithelial cells (Huang, Brangwynne et al. 2005). Those data suggest that PAI-1 renders detached clusters invasive.

Our data indeed suggest that PAI-1 generates actomyosin contractility to promote cell movement without a specific direction in the 2D model. Meanwhile, PAI-1-induced cell migration in the 3D invasion model seems to gain directionality when cells move collectively as clusters inside which they can rotate. This could explain in a way the random movement that PAI-1 promoted in our 2D system. Those observations support the pertinence of our 3D invasion model to study PAI-1 role in cell detachment and invasion from MCTS 3D spheroids of non-invasive cancer cells. Altogether our data demonstrate PAI-1 capacity to induce

detachment of cell clusters that migrate collectively away from spheroids into the 3D surrounding matrix.

PAI-1 cell clustering & detachment in 2D model

The role of PAI-1 in cell detachment and cell clustering was assessed using a 2D model in which spread islands of cells or individualized cells were seeded on pro-adhesive matrices. Concerning the role of PAI-1 in supporting cell clustering, we showed that PAI-1 addition to isolated MCF-7 or HCT116 cells in 2D cell culture, rapidly induced their aggregation with each other to become organized in 3D multilayer clusters on control coating (BSA) (Figure 2A & 2B) as well as on collagen and fibronectin coatings (Figure 2D) strongly suggesting that PAI-1 induced cells grouping to form clusters (cell clustering), independently of the matrix. However, HCT116 p21^{-/-} cells, deprived of E-Cadherin cell-cell junctions, were not able to organize in 3D multilayer clusters when treated with PAI-1, although they were more grouped in comparison with untreated cells (Figure 2A). This suggests that PAI-1-mediated clustering or grouping of cells as 3D multilayers clusters requires intercellular junctions. We also found that E-cadherin and tight junctions (ZO-1) were maintained at cell-cell junctions in PAI-1-induced clusters (Figure 3F, 3G, 3J), supporting thus our observation.

To study PAI-1 de-adhesive activity, we treated epithelial cells seeded on pro-adhesive matrices as collagen and fibronectin with PAI-1 after (24h) complete spreading, PAI-1 disrupted spread islands organization and forced cells to aggregate in multilayers clusters of identical structure to those observed both in PAI-1 clustering effect and in the 3D model. This strongly suggests that PAI-1 effects disrupt cell-cell and cell-matrix adhesions, independently of the underlying matrix (Figure 2F, 2G, and Supplementary videos 8 & 9 and supplementary images in Figure S3).

Although previously was shown that soluble PAI-1 didn't have a significant effect to maintain blebbing of invasive cells compared to PAI-1 coating (Cartier-Michaud, Malo et al. 2012), in our model, whether PAI-1 was soluble or coated on cover-slips, it induced identical multi-layered clusters from non-invasive cells (Figure 2D, 2E), indicating that soluble or matrix-bound PAI-1 forms that are naturally found in the microenvironment, were able to induce cells clustering. The differences could refer to cell context differences in addition to

the difference in the final phenotypes and the involved PAI-1 underlying mechanism. Altogether our data indicate that PAI-1 has a de-adhesive and a clustering effect on epithelial non-invasive cancer cells and allows their reorganization in multicellular clusters harbouring multilayers spheroid-like shape.

PAI-1: uPA-independent/ LDL receptor-dependent

- uPA-independent

Previous reports have explored PAI-1 role in complete cells detachment and suspension from the substrate as a final result and shown that PAI-1 capacity to completely detach cells from the substrate could involve or not uPA and that is dependent on the type of cell adhesive receptors (uPAR, integrins or both) and their expression level on the cell surface. Loskutoff and colleagues explored cell detachment. They showed that when cells were seeded on Vitronectin (an ECM molecule) they bind it through uPAR alone (e.g., U937 cells) and in this case, integrins are not engaged. PAI-1 can completely detach those cells by directly competing with uPAR for the binding site in the SMB domain of Vitronectin (Deng, Curriden et al. 2001), (Czekay, Aertgeerts et al. 2003). Since, as far as we know, neither PAI-1 nor uPAR bind to other matrix proteins than Vitronectin, this mechanism of competitive displacement is specific for cells growing on Vitronectin and will not affect cells growing on other matrices such as fibronectin or Collagen-I. Opposite to those results, we show that PAI-1 alone was able to detach cells spread on collagen and fibronectin (Figure 2D, 2F, 2G) suggesting a different mechanism involved in PAI-1 de-adhesive activity in our conditions. In agreement, when cells adhere to Vitronectin or collagen or fibronectin either through integrins alone (e.g MCF-7 cells which express extremely low quantity of uPAR) or through integrins and uPAR (i.e., most other cells), this disengagement of uPAR by PAI-1 is not sufficient to detach the cells, since cells remain firmly anchored via integrins. Sequential addition of uPA and PAI-1 promoted cell detachment (Deng, Curriden et al. 2001), (Czekay, Aertgeerts et al. 2003). Interestingly, PAI-1 cell detachment required direct interaction between uPAR and integrins but didn't require their internalization by LDL receptors (LRP/vLDLR) as R76E mutant PAI-1 that does not bind those receptors or blocking anti-bodies against those receptors did not affect cell detachment induced by PAI-1, showing that PAI-1 in those conditions induced cell detachment in uPA/uPAR dependent and LDL-R independent manner (Czekay and Loskutoff 2009). Considered as non-invasive cancer cell lines, MCF-7 and HCT-

116 cells express very few of uPA and uPAR protein compared to invasive cells (Holst-Hansen, Johannessen et al. 1996), (Deng, Curriden et al. 2001), (Fanny Tomas, unpublished results). On the other hand, they express alpha 5 integrins (Deng, Curriden et al. 2001), (Jia, Starodub et al. 2013). Opposite to previous reports, we show that PAI-1 alone had a de-adhesive activity able to induce the formation of multi-layered clusters from MCF-7 (mainly integrins on its surface) or HCT116 (uPAR, integrins on its surface) on fibronectin and collagen-I and that involved LDL-R receptors (Figure 2, Figure 4A). This suggests that PAI-1 independently of uPA was able to detach cells. Although an implication of uPA/uPAR should be verified, our observations, suggest that PAI-1 uses a different mechanism to induce cell detachment and to form 3D multi-layered clusters.

- *LDL-R/ROCK/JAK dependent*

In previous reports, LRP as an endocytosis receptor of many ligands and as signalling molecule, known to be involved in the regulation of cancer cell migration and invasion. LRP was shown to induce thyroid carcinoma cell invasion through regulating cytoskeletal organization and adhesion complex turnover by activating ERK and inhibiting JNK pathways (Dedieu, Langlois et al. 2008), (Langlois, Perrot et al. 2010). Similar results have shown that vLDL receptor mediated uPA/PAI-1-induced ERK phosphorylation was able to induce cell migration (Webb, Thomas et al. 2001). However, we show that PAI-1 de-adhesive activity does not require ERK activation as MEK kinase inhibitor did not have a significant effect on PAI-1 cluster formation (Figure 4B) suggesting that even if PAI-1 action requires vLDLR or LRP, it doesn't depend on ERK activation that could be potentially stimulated through one or both receptors. Furthermore, previous reports have shown that cell detachment by PAI-1 was LRP/vLDLR independent as blocking PAI-1 and LRP/vLDLR interaction did not inhibit PAI-1-induced detachment (Czekay and Loskutoff 2009). On the contrary, we show that Mutant R76E-PAI-1 failed to detach cells to induce 3D multilayer cluster formation from spread cells on Collagen or Fibronectin (Figure 4A). Although a further investigation is required to distinguish the involvement of those receptors, our results indicate strongly that PAI-1 action involves LRP and/or vLDLR receptor, independently of ERK activation.

Of note, PAI-1 action in our study is likely LDL-R dependent and uPA-independent. In accordance to previously mentioned reports, invasive SW620 cells blebbing induced by

immobilized PAI-1 was not dependent on LRP receptor but uPA/uPAR-dependent (Cartier-Michaud, Malo et al. 2012). These data support our hypothesis that different cell context (invasive vs. non-invasive features) and different final phenotypes could have completely different mechanisms induced by the same molecule. Meanwhile, regardless of the final phenotype induced by PAI-1, it seems that PAI-1 action is either dependent on LRP/vLDLR receptors or on uPA/uPAR, thus supporting our conclusions.

In our screening for signalling pathways, which may mediate the de-adhesive effect of PAI-1 downstream of LDL receptors, we further determined that the inhibition of Src, PI3K and MEK kinases had no effect on the formation of PAI-1 induced 3D clusters, whereas inhibition of JAK and ROCK kinases greatly reduced it both on BSA (control coating) and on collagen coatings (Figure 4B, Figure 4C), thus strongly suggesting that PAI-1 activity is ROCK/JAK dependent.

In coherence with our results and hypotheses, it was previously reported that the different conformations of PAI-1 (active, latent, cleaved) can bind LRP1 and activates JAK/STAT1 dependent migration in chemotaxis and monolayer wound healing assays. PAI-1 induced LRP/JAK/STAT migration independently of uPA activity or Vitronectin binding as cleaved form of PAI-1 that neither binds to Vitronectin nor inhibits uPA, activates this signalling (Degryse, Neels et al. 2004), (Kamikubo, Neels et al. 2009), (Hou, Zheng et al. 2002). Moreover, secreted PAI-1 by Glioma cells increased mast cells exocytosis and recruitment to glioma and via LRP/STAT3 activity (Roy, Coum et al. 2015). In addition, RhoA/ROCK and/or JAK/STAT signalling pathways are known to be involved in cell migration and invasion. For example, Rho Family GTPase activity has been connected with enhanced expression of JAK/STAT signalling (Loucks, Le et al. 2006), (Pelletier, Duhamel et al. 2003). Notably, Cancer cell-derived GFs and cytokines can promote α 5-integrin-mediated activation of ROCK, as well as activation of JAK1 and induction of pSTAT3 in stromal cells, thus converting them into CAFs. These CAFs are able to contract collagen and also make passageways in the surrounding matrix by JAK1-ROCK- controlled actomyosin contractility, thereby enabling cancer cell invasion (Cirri and Chiarugi 2011), (Gaggioli, Hooper et al. 2007), (Sanz-Moreno, Gaggioli et al. 2011). Those reports are in line with our results that indicate an involvement of LDL-R, ROCK and JAK kinases in PAI-1 induced cluster migration. We confirmed our results by evaluating the importance of JAK and ROCK kinases

activities in the invasive role of PAI-1 in 3D collagen matrix. Treatment of spheres with either JAK or ROCK inhibitors significantly decreased PAI-1-induced invasion and cluster formation in a 3D collagen matrix (Figures 4D and E). Altogether our results suggest that LDL-R/ ROCK & JAK are involved in both PAI-1 de-adhesive effect and cluster invasion in 3D model.

PAI-1 induces actomyosin contractility

Clusters of tumour cells exhibit considerable flexibility to invade surroundings tissues. We showed that PAI-1 induced invasive clusters that maintained cell-cell junctions and their actin network was distinctly delocalized to the cluster periphery (Figure. 3F, 3G, 3I, 3H). In agreement, addition of PAI-1 rapidly and transiently changes cell morphology by reorganizing actin skeleton during single cell migration (Degryse, Neels et al. 2004). Thus we studied the involvement of actomyosin contractility in PAI-1 action by evaluating the phosphorylation and the localization of MLC2 in myosin II downstream of ROCK as cortical P-MLC2 staining indicative of its activation was observed previously (Sahai, Garcia-Medina et al. 2007), (Gaggioli, Hooper et al. 2007). We showed that P-MLC2 preferentially concentrated in cells located at the periphery of cluster induced by PAI-1 (Figure 5A&5B). This result indicates that PAI-1 increases actomyosin contractility especially at the cluster periphery. In addition to our result showing that inhibition of ROCK and JAK greatly reduced PAI-1 cluster formation (Figure 4B, Figure 4C), we further showed that treatment of PAI-1 induced clusters with ROCK inhibitor increased their surface area and reduced their circularity indicating that clusters were more flattened and elongated following ROCK inhibition (Supp. Figure S4). This suggests an involvement of contractility in cluster formation. Similarly, using CAAX-GFP-labelled cells forming the MCTS spheroid in the 3D invasion model, we observed that labelling was intensified in groups of cells on the spheroid periphery preceding their detachment to generate invading clusters (Figure 5D, Supp. videos 10, 11, 12 and 13). We showed that the inhibition of ROCK or JAK kinases, known to induce contractility, highly reduced cluster 3D invasion, supporting the notion that actomyosin contractility was involved in PAI-1 action. Aznar and colleagues have previously shown that RhoA positively controls STAT3 full activation (Aznar, Valeron et al. 2001). Similarly, ROCK and JAK1 kinases were reported to be closely linked with the induction of actomyosin contractility in order to generate tumour aggressive phenotype through positively modulating the activity of the transcription factor STAT3 (Sanz-Moreno, Gaggioli et al. 2011). In

agreement, our results indicate that STAT3 is phosphorylated and translocated to the nucleus indicative of its activation following PAI-1 treatment. This P-STAT3 activation was drastically reduced following ROCK or JAK kinases inhibition, thus indicating that both ROCK and JAK regulate p-STAT3 activation under PAI-1 induction (Figure 5F, 5G, 5E). Our data strongly suggests that PAI-1 involves ROCK/MLC2 and JAK/STAT signalling pathway in the regulation of actomyosin contractility. A further issue could be verified concerning a direct link between JAK1 and P-MLC2 as it was previously shown that JAK1 kinase could also regulate both P-MLC2 and P-STAT3 (Sanz-Moreno, Gaggioli et al. 2011).

Interestingly, it was previously reported that cells which migrate as clusters often behave in a cooperative manner where force and contractility is mediated via cell-cell junctions making the cohort behave as one big cell (Friedl and Alexander 2011), (Hidalgo-Carcedo, Hooper et al. 2011). Furthermore, actomyosin contractility is tightly regulated at cell-cell contacts in collectively migrating cells. Silencing of RhoA inhibits the formation of cell-cell contacts while RhoA overexpression, depletion of the RhoGAPs myosin IXA or p190RhoGAP or depletion of RhoE, which antagonizes ROCK-driven actomyosin contractility, lead to cell scattering and impaired collective migration of different epithelial cells (Desai, Aryal et al. 2004), (Hidalgo-Carcedo, Hooper et al. 2011), (Omelchenko and Hall 2012). A very recent report showed a role for actomyosin contractility to prevent the snail-dependent EMT disassembly of cell-cell junctions (Weng and Wieschaus 2016). Those findings suggest that a fine tuning of actomyosin contractility could regulate the maintenance or the break-down of cell-cell junctions. In other words, PAI-1 could induce actomyosin contractility to a suitable threshold and consequently modulates cell-cell junctions to induce cluster detachment and their migration into the 3D surrounding matrix. A similar hypothesis was validated in a drosophila model to study epithelial cells division. During cytokinesis, an increase in actomyosin contractility is necessary to reach a threshold of forces that enable cell-cell junctions disassembly and to allow the formation of new junctions in daughter cells. This increase in contractility was regulated by Septin, a GTP binding protein, involved in cytokinesis (Founounou, Loyer et al. 2013). Interestingly, Septin was also described to control actomyosin contractility and membrane retraction during blebbing of amoeboid mouse T cells. Those findings could prompt us to imagine a similar role for PAI-1-induced contractility in triggering cluster detachment and migration.

Other hypotheses can be proposed for actomyosin contractility mediated PAI-1 invasion. As previously described, Wiskott-Aldrich Protein Family-3 (WASF3) is a known regulator of actomyosin contractility to induce cancer invasion (Takenawa and Suetsugu 2007), (Teng, Mei et al. 2014). Importantly, JAK/STAT3 signalling induced WASF3 (Teng, Ghoshal et al. 2013). Thus we can assume that an induction of WASF3 protein could also contribute to contractility-induced invasion promoted by PAI-1, in a ROCK/MLC2/JAK/STAT3 dependent manner. Interestingly, different cytokines such as IL-6, LIF and oncostatin M were reported to promote invasion by inducing contractility via JAK/ROCK/STAT3 pathway (Sanz-Moreno, Gaggioli et al. 2011). However, Oncostatin M was the only inflammatory cytokine compared to LIF and IL-6 to highly increase PAI-1 production and secretion (Demyanets, Kaun et al. 2007), (Sanz-Moreno, Gaggioli et al. 2011). We can imagine here that as PAI-1 is downstream of OSM, it could contribute to the increase of cell invasion involving the same signalling pathway.

Cell-cell junctions mediates contractility in cells migrating as clusters, thus making the cluster behave as one big cell (Friedl and Alexander 2011), (Hidalgo-Carcedo, Hooper et al. 2011). In coherence, we show that PAI-1- induced clusters maintained cell-cell junctions (Figure 3F, 3G, 3J) together with actin network and P-MLC2 which were distinctly delocalized to the cluster periphery (Figure 3I, 3H) indicating an induction of cortical actomyosin contractility. In addition, Orgaz and colleagues reported that MMP-9 non-catalytic function regulates rounded amoeboid cells to induce actomyosin contractility driven by RhoA/ROCK and JAK/P-STAT3 (Orgaz, Herraiz et al. 2014). Together, those data suggest that PAI-1 detached clusters could behave as single amoeboid cell and that cells at the periphery of the cluster increase their actomyosin contractility to mimic the cortical contractility observed in amoeboid single cells, taking into consideration signalling pathways similarity.

As PAI-1 is known to mediate integrins internalization (Czekay, Aertgeerts et al. 2003), it is also possible to imagine that PAI-1 action could involve integrins, as integrins were previously described to induce a similar signalling to the one induced by PAI-1, facilitating cell invasion (Cirri and Chiarugi, 2011; Gaggioli et al., 2007; Sanz-Moreno et al., 2011).

We evidence here that PAI-1 generates mechanical forces by actomyosin-driven contraction that contribute to separation of cell clusters from the sphere mass, thus proposing an energetically low-cost mechanism as eventually it saves cancer cells energy that could be spent potentially for epithelial and mesenchymal conversions.

It is worth mentioning that even though active PAI-1 is routinely cleared from the *in-vivo* extracellular environment, latent and cleaved (that does not inhibit uPA or does not bind to Vitronectin) species of PAI-1 could remain embedded in the matrix. Interestingly, these different forms of PAI-1 can bind to LRP receptor independently of uPA, to induce JAK/STAT signalling and cell migration (Degryse, Neels et al. 2004), (Kamikubo, Neels et al. 2009), (Hou, Zheng et al. 2002) thus suggesting that the signalling pathway proposed by our study could be generalized and be constantly induced by the different forms of PAI-1 secreted in the tumour microenvironment despite the internalization of its active form. Furthermore, we do not exclude a biomechanical induction of cell contractility by PAI-1 through LDL-receptor with/without the implication of integrins or another co-receptor as proposed and described previously. (Cartier Michaud et al, 2012), (Wang et al, 1995).

Finally, we propose a model in which PAI-1 that is highly secreted in the tumour stroma of many cancers, to be the first extracellular secreted protein that promotes a non-EMT epithelial cluster detachment and invasion by directly inducing actomyosin contractility of non-invasive epithelial cancer cells present in the primary tumour in a ROCK/MLC2/JAK/STAT3 dependent manner (Figure 5H).

PAI-1 was shown to promote already invasive cancer cells migration. However, our work unravel a novel function for the extracellular protein PAI-1 that extends its action to influence on non-invasive epithelial cancer cells promoting their detachment and invasion in a collective manner (**Figure 12**), probably at early stages of tumour development. This finding strongly supports the correlation of high PAI-1 levels with poor prognosis and insists firmly to consider this novel signalling axis induced by PAI-1 as a therapeutic target in order to prevent epithelial cancer metastatic invasion at primary tumour level.

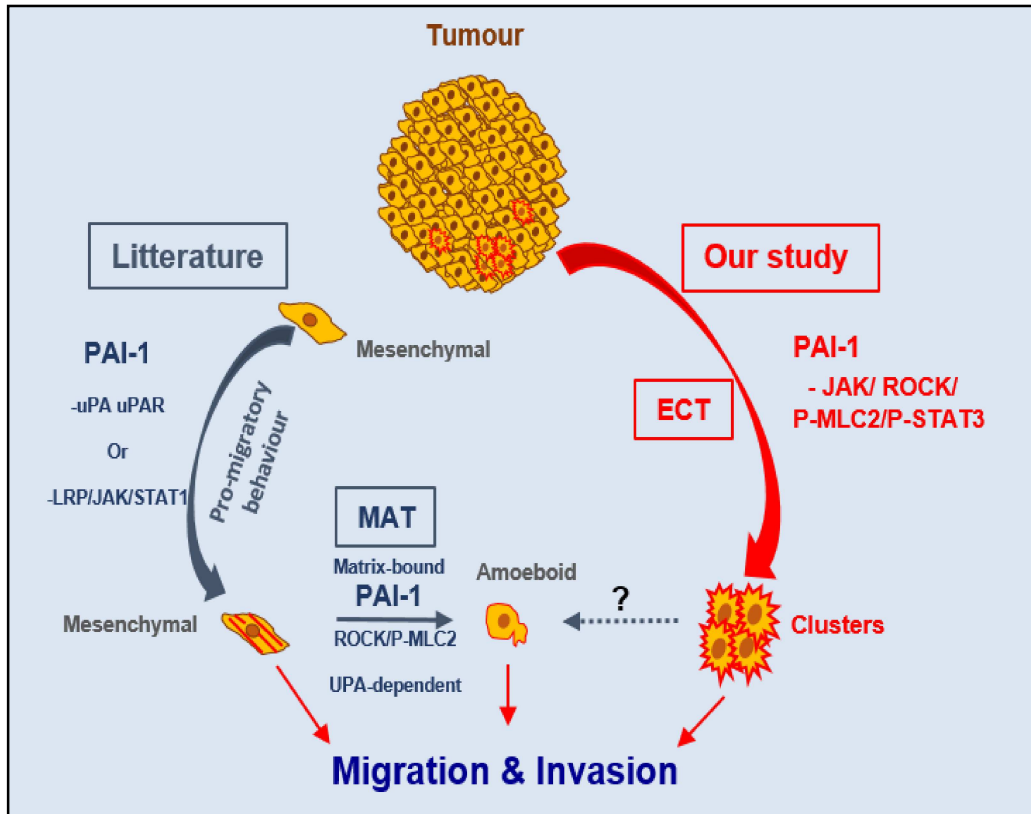


Figure 12. Diverse roles of extracellular PAI-1 in cell migration.

PAI-1 plays a pro-migratory role of single cells both in its soluble or matrix-bound forms. Soluble PAI-1 increased mesenchymal cells migration rates through uPA/uPAR turnover or by activating JAK/STAT signalling through LRP (Chazaud, Ricoux et al. 2002), (Degryse, Neels et al. 2004). Matrix-bound PAI-1 promotes the transition of a mesenchymal cell into an amoeboid-like state (MAT) (Cartier-Michaud, Malo et al. 2012). Soluble PAI-1 promotes collective cluster detachment and migration of epithelial non-invasive cells via JAK/ROCK/MLC2/STAT3 dependent manner (Our study).

PUBLICATIONS

1- Paper to be submitted:

PAI-1 promotes tumour cell detachment in clusters leading to collective cell invasion through a ROCK and JAK dependent signalling.

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2- Paper submitted at *eLife Journal*.

TP53 drives invasion through expression of its $\Delta 133p53\beta$ variant.

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3- Paper to be submitted: *Oncotarget Journal*

Chemotherapy targeting invasive and tetraploid soft tissue sarcomas cells lines

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Scientific Conferences:

- 1- **Young Researchers Days conference:** 03-04 november 2015 – Cité Universitaire, Paris, FRANCE

Authors: Samer ABDALLAH, Georgia BARLOVATZ, Véronique GIRE* and Pierre ROUX*

Poster title: Extracellular PAI-1 promotes epithelial cancer cell invasion through cell clusters detachment via ROCK & Jak-dependent signaling

- 2- **Integrated Mechano-Chemical Signals in Cancer Invasion conference:** 16-20 October 2015 - Saint-Paul-de-Vence, Nice, FRANCE

Authors: Samer ABDALLAH, Georgia BARLOVATZ, Pierre ROUX and Véronique GIRE

Poster title: Extracellular PAI-1 promotes epithelial cancer cell invasion through cell clusters detachment via ROCK & Jak-dependent signaling

3- “ **P53 isoforms meeting**” conference: 20-23 Septembre 2015 - Aix en provence, FRANCE

Authors: Fanny TOMAS, Samer ABDALLAH, Nikola ARSIC, Pierre ROUX and Véronique GIRE.

Poster title: $\Delta 133\beta$ isoform regulates cell proliferation in primary human fibroblasts

4- EMBO conference “ **Centrosomes and Spindle Pole Bodies**” : 30 september-3 october 2014, Lisbonne, Portugal

Authors: A. Aubusson-Fleury; M. Lemullois; H. Bengueddah; S. Abdallah; L. Shi; J. Cohen; F. Koll

Poster Title: Transition zone: the sequential assembly of its components parallels its dual role in basal body anchoring and ciliary function

5- **Cilia conference**: 18-21 november 2014, Lisbonne, Portugal

Authors: A. Aubusson-Fleury; M. Lemullois; H. Bengueddah; S. Abdallah; L. Shi; J. Cohen; F. Koll

Poster Title: Transition zone: the sequential assembly of its components parallels its dual role in basal body anchoring and ciliary function

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RESUME

L'invasion de la matrice extracellulaire est le premier obstacle que les tumeurs solides rencontrent lors de leur dissémination métastatique. Il existe deux mécanismes principaux: 1) la transition épithélio-mésenchymateuse au cours de laquelle les cellules épithéliales, à l'échelle individuelle, perdent leur cohésion intercellulaire et acquièrent des capacités de motilité pour quitter leur site d'origine ; 2) l'invasion collective dans laquelle des cellules en groupe coopèrent pour migrer collectivement en se frayant un passage à travers le microenvironnement. Dans les modèles murins, on sait maintenant que des groupes ou agrégats de cellules tumorales circulantes issus du détachement de tumeurs primaires présentent une capacité accrue à coloniser des organes à distance. Cependant, les facteurs et mécanismes sous-jacents conduisant au détachement des cellules tumorales en groupe sont peu connus. Pour étudier ces mécanismes, nous avons utilisé des sphéroïdes tumoraux, modèle expérimental tri-dimensionnel (3D), qui mime le microenvironnement ainsi que les caractéristiques morphologiques et fonctionnelles de la tumeur primaire. Nos résultats indiquent que l'inhibiteur de l'activateur du plasminogène (PAI-1), une protéine matricellulaire trouvée en forte concentration dans les cancers invasifs, favorise le détachement en groupe et l'invasion collective des cellules du cancer du sein (lignée cellulaire MCF7) et du colon (lignée cellulaire HCT116) organisées en sphéroïdes tumoraux. PAI-1 a des propriétés dé-adhésives sur des cellules étalées en monocouche sur des matrices pro-adhésives et génère l'agrégation des cellules entre-elles. Ces cellules conservent leurs caractéristiques épithéliales malgré une modification importante de la dynamique du réseau d'actine. Nos résultats suggèrent que les récepteurs cellulaires de la famille des lipoprotéines de basse densité (LDL-R) sont impliqués dans les propriétés dé-adhésives de PAI-1. Dans la cellule, la transduction du signal passe par les kinases ROCK (kinase associée à la GTPase RhoA) et Janus (JAK), ce qui conduit à une phosphorylation de la chaîne légère de la myosine II (MLC2), nécessaire pour l'activité contractile de la myosine et des facteurs de transcription STAT3. En outre, ROCK, un régulateur connu de la contraction du cytosquelette d'actomyosine, est également impliqué dans l'activation de STAT3. L'inhibition de ROCK ou JAK rétablit l'adhésivité des cellules en 2D et réduit leur migration en 3D. Nos données suggèrent que PAI-1 génère des zones de forte activité contractile membranaire dans les cellules en périphérie de la tumeur via ROCK-MLC2 et JAK-STAT, ce qui promeut le détachement de groupe de cellules hautement invasives. Ce nouvel axe de signalisation fonctionnelle de PAI-1 représente une cible anti-métastatique potentielle.

ABSTRACT.

Invasion of the extracellular matrix is the first obstacle that solid tumours encounter during their metastatic dissemination. There are two main mechanisms: 1) epithelial to mesenchymal transition wherein epithelial cells lose their intercellular cohesion and convert to individual migratory behaviours to escape their point of origin; 2) invasion in a collective manner, in which a group or cluster of cohesive cancer cells detaches from the tumour mass and progressively, pushes its way through the microenvironment. It is now known that circulating tumour cell clusters may result from the evasion of cohesive small groups of cells from tumours and such tumour cell clusters display an increased propensity to colonize distant organs in mouse models. However, the extracellular factor/s and the underlying mechanism that enable cell detachment, as clusters are largely unknown. To study the process of tumour cell detachment and invasion, we used a three-dimensional (3D) multicellular tumour spheroid (MCTS) model, which mimics the microenvironment as well as morphological, functional and symmetric geometry features of the primary tumour. Our results strongly indicate that the plasminogen activator inhibitor-1 (PAI-1), a matricellular protein found in high concentration at the invasive front of most cancers, promotes cancer cell cluster detachment and a collective invasion phenotype within MCTS of breast cancer MCF7 and colon cancer HCT116 cell lines. We found that PAI-1 has a de-adhesive effect which induced a multilayered cell clustering of cells spread out on a pro-adhesive matrices in 2D monolayer cultures. Cells retained their epithelial characteristics and membrane-localized E-cadherin despite significant modification of actin dynamics. PAI-1 functions as a de-adhesive molecule most likely involved low-density lipoprotein receptors at the cell surface. We report that the downstream intracellular events may be mediated through the Rho-associated kinase (ROCK) and Janus kinases (JAK) signalling pathway, resulting in phosphorylation of either myosin light chain 2 (MLC2), which is required for myosin II-mediated contractility or STAT3 transcription factors. In addition, ROCK, a known contributor to actomyosin contractility is involved in STAT3 phosphorylation and activation. Inhibition of ROCK and JAK restored adhesion of cells on 2D substratum and reduced their migration/invasion within 3D MCTS model. Our data support a model in which PAI-1 generates actomyosin contractility and high membrane activity at the tumour periphery in a JAK-STAT/ROCK-MLC2 dependent manner promoting the detachment of highly invasive cell clusters. This novel axis of functional signalling of PAI-1 is a potential anti-metastasis target.