

# Cooperation of MT1-MMP and receptor tyrosine kinase signalling in cancer cell invasion

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## Contents

|  |    |
|--|----|
| ORIGINAL PUBLICATIONS .....                                    | 6  |
| ABBREVIATIONS .....  | 7  |
| ABSTRACT .....   | 9  |
| REVIEW OF THE LITERATURES .....                                | 10 |
| INTRODUCTION .....   | 10 |
| 1. Tumour microenvironment .....                               | 11 |
| 1.1 Extracellular matrix (ECM) .....                           | 11 |
| 1.1.1 Structure of ECM.....                                    | 11 |
| 1.1.1.1 Interstitial matrix .....                              | 11 |
| 1.1.1.2 Basement membrane (BM) .....                           | 12 |
| 1.1.2 ECM Remodelling in Cancer .....                          | 13 |
| 1.2 Communication between cancer cells and stromal cells ..... | 14 |
| 1.2.1 Cancer-associated fibroblasts (CAF) .....                | 14 |
| 1.2.2 Angiogenesis and lymphangiogenesis .....                 | 14 |
| 1.2.2.1 Cancer cell metastasis through circulation.....        | 15 |
| 1.2.3 Infiltrating immune cells .....                          | 16 |
| 2 Cancer cell invasion .....                                   | 16 |
| 2.1 Single-cell invasion .....                                 | 17 |
| 2.1.1 Amoeboid-type invasion .....                             | 18 |
| 2.1.2 Mesenchymal-type invasion.....                           | 18 |
| 2.2 Multicellular invasion .....                               | 19 |
| 2.2.1 Multicellular streaming .....                            | 19 |
| 2.2.2 Collective cell invasion .....                           | 20 |
| 2.3 Plasticity of cancer cell invasion .....                   | 21 |
| 2.3.1 Collective-to-single cell transition.....                | 21 |
| 2.3.1.1 Epithelial-to-mesenchymal transition (EMT) .....       | 21 |
| 2.3.2 Mesenchymal-to-amoeboid transition.....                  | 22 |
| 3 Human protein kinases/Human kinome .....                     | 23 |
| 3.1 Protein kinases in cancer.....                             | 24 |
| 4 Receptor tyrosine kinases (RTKs) in cancer.....              | 25 |
| 4.1 Fibroblast growth factor receptor family .....             | 25 |
| 4.1.1 FGF signalling .....                                     | 26 |
| 4.1.2 FGF signalling in cancer .....                           | 27 |
| 4.1.2.1 Gain-of-function mutations .....                       | 27 |
| 4.1.2.2 Single nucleotide polymorphism (SNP) .....             | 28 |
| 4.1.2.3 Chromosomal translocations .....                       | 28 |
| 4.1.2.4 Gene amplification and overexpression.....             | 29 |
| 4.1.2.5 Aberrant FGF signalling .....                          | 29 |
| 4.2 Eph receptor family .....                                  | 29 |
| 4.2.1 Eph signalling.....                                      | 30 |
| 4.2.2 EphA2 in cancer .....                                    | 31 |
| 4.2.2.1 Overexpression .....                                   | 31 |
| 4.2.2.2 Ligand-independent signalling .....                    | 32 |
| 4.2.2.3 SNPs and Mutations.....                                | 33 |
| 4.3 FGF and Eph signalling in cancer cell invasion.....        | 33 |
| 4.3.1 FGF signalling in cancer cell invasion.....              | 33 |
| 4.3.2 Eph signalling in cancer cell invasion.....              | 34 |
| 5 Matrix Metalloproteinases .....                              | 36 |
| 5.1 MT-MMPs.....   | 36 |

|         |  |    |
|---------|--|----|
| 5.1.1   | MT-MMP activity.....   | 38 |
| 5.1.2   | Regulation of MT-MMP activity.....   | 40 |
| 5.1.2.1 | Transcriptional regulation.....  | 40 |
| 5.1.2.2 | MMP inhibitors.....  | 41 |
| 5.1.2.3 | Intracellular trafficking and cell-surface localization of MT-MMPs.....  | 41 |
| 5.2     | Functions of MT1-MMP.....  | 43 |
| 5.2.1.1 | MT1-MMP function in development.....   | 43 |
| 5.2.1.2 | MT1-MMP in cancer cell invasion and metastasis.....  | 44 |
|         | AIMS OF THE STUDY.....   | 46 |
|         | MATERIALS AND METHODS.....   | 47 |
| 1       | Methods used in this study.....  | 47 |
| 2       | Cell culture and reagents (I, II, III).....  | 47 |
| 3       | Antibodies, chemicals, and growth factors (I, II, III).....  | 48 |
| 4       | Chemicals and growth factors.....  | 49 |
| 5       | DNA and transfection (I, II, III).....   | 50 |
| 6       | cDNA mutagenesis assay (I, III).....   | 51 |
| 7       | siRNAs and shRNAs (I, II, III).....  | 52 |
| 8       | RNA extraction and quantitative real-time PCR (I, II, III).....  | 52 |
| 9       | Gelatine zymography (I).....   | 53 |
| 10      | Immunoblotting, immunoprecipitation, and mass spectrometry analyses (I, II, III).....  | 53 |
| 11      | Cell surface biotinylation.....  | 53 |
| 12      | Immunofluorescence (I, II, III).....   | 54 |
| 13      | 3D type I collagen invasion and invasive growth analyses (I, II, III).....   | 54 |
| 14      | Mouse tumour growth analysis (II, III).....  | 54 |
| 15      | Histologic analyses and immunohistochemistry (II, III).....  | 54 |
| 16      | FGFR4 allele genotyping analysis (I, II).....  | 55 |
| 17      | Rho-GTPase activity assay (III).....   | 55 |
| 18      | Cell detachment and cell-cell repulsion analysis (III).....  | 55 |
| 19      | Statistical analysis (I, II, III).....   | 56 |
|         | RESULTS AND DISCUSSION.....  | 57 |
| 1       | Identification of receptor tyrosine kinases, FGFR4 and EphA2, as unique regulators of proinvasive MT1-MMP activity (I, II, III).....       | 57 |
| 2       | FGFR4 polymorphism acts as an activity switch of MT1-MMP-mediated cancer cell invasion (I, II).....  | 57 |
| 2.1     | The FGFR4-R388 risk variant increases MT1-MMP levels by reducing lysosomal degradation of MT1-MMP (I).....                                 | 58 |
| 2.2     | The FGFR4-R388 risk variants increases MT1-MMP phosphorylation and endosomal stabilization (I).....  | 58 |
| 2.3     | MT1-MMP and FGFR4-R388 activate and MT1-MMP and FGFR4-G388 suppress each other (I).....  | 59 |
| 2.4     | The FGFR4-G388R risk variant promotes MT1-MMP-mediated collagen degradation (I, II).....   | 60 |
| 3       | FGFR4 and MT1-MMP are co-expressed at invasive edge in human tumours (II).....   | 61 |
| 3.1     | The FGFR4-R388 variant associates with MT1-MMP mRNA expression in high grade (Grade 3) breast carcinomas (II).....                         | 61 |
| 3.2     | MT1-MMP and FGFR4 are co-expressed in stroma/tumour border and in invasive front of breast, lung, colon, and prostate carcinomas (II)..... | 61 |
| 4       | FGFR4 regulates MT1-MMP-dependent ECM degradation and tumour progression in 3D collagen matrix and <i>in vivo</i> (II).....                | 62 |

|     |   |    |
|-----|---|----|
| 4.1 | FGFR4-R388 and MT1-MMP induce epithelial-to-mesenchymal transition (EMT) (II)   | 62 |
| 4.2 | Endogenous FGFR4/MT1-MMP axis controls prostate carcinoma cell differentiation, extracellular matrix degradation, and EMT <i>in vivo</i> (II) | 63 |
| 5   | EphA2 cleavage by MT1-MMP triggers single cancer cell invasion via homotypic cell repulsion (III)   | 64 |
| 5.1 | EphA2 and MT1-MMP are co-expressed in invasive breast carcinoma cells (III)   | 64 |
| 5.2 | MT1-MMP cleaves EphA2 to modulate receptor localization and cell junctional properties (III)  | 65 |
| 5.3 | MT1-MMP cleaves EphA2 at fibronectin type-III domain <i>in cis</i> on the cell surface (III)  | 66 |
| 5.4 | MT1-MMP-dependent EphA2 processing triggers cell-cell repulsion (III)   | 67 |
| 5.5 | Prominent EphA2 cleavages promote RhoA activation and single-cell invasion within 3D collagen matrices (III)                                  | 68 |
| 5.6 | EphA2 cleavage promotes single cell dissemination <i>in vivo</i> (III)  | 69 |
|     | CONCLUSIONS AND PERSPECTIVES  | 70 |
|     | ACKNOWLEDGEMENTS  | 72 |
|     | Reference List  | 74 |

## ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to their Roman numerals in the text.

- I. Sugiyama N., Varjosalo M., Meller P., Lohi J., Chan K.M., Zhou Z., Alitalo K., Taipale J., Keski-Oja J. and Lehti K. FGF receptor-4 polymorphism acts as an activity switch of a membrane-type-1 matrix metalloproteinase-FGFR4 complex. *Proc Natl Acad Sci U S A.* 2010; 107, 15786-15791.
- II. Sugiyama N., Varjosalo M., Meller P., Lohi J., Hyytiäinen M., Kilpinen S., Ingvarsen S., Engelholm L. H., Kallioniemi O., Alitalo K., Taipale J., Keski-Oja J. and Lehti K. FGF receptor-4 regulates tumor invasion by coupling FGF-signaling to extracellular matrix degradation. *Cancer Res.* 2010; 70, 7851-7861.
- III. Sugiyama N\*, Gucciardo E\*, Tatti O, Varjosalo M, Hyytiäinen M, Gstaiger M and Lehti K, EphA2 cleavage by MT1-MMP triggers single cancer cell invasion via homotypic cell repulsion. *J. Cell Biol.* 2013; 201, 467-84. \* equal contribution

## ABBREVIATIONS

|            |  |
|------------|--|
| 2D         | two-dimensional  |
| 3D         | three-dimensional  |
| ADAM       | a disintegrin and metalloproteinase                            |
| ADAMTS     | a disintegrin and metalloproteinase with thrombospondin motifs |
| ALK        | activin-like kinase  |
| BM         | basement membrane  |
| B-RAF      | v-raf murine sarcoma viral oncogene homolog B1                 |
| CAF        | cancer-associated fibroblast                                   |
| CIL        | contact inhibition of locomotion                               |
| CoA        | co-attraction  |
| E-Cadherin | epithelial cadherin  |
| ECM        | extracellular matrix   |
| EEA1       | early endosomal antigen 1                                      |
| EGF        | epidermal growth factor  |
| EGFR       | epidermal growth factor receptor                               |
| EMT        | epithelial-to-mesenchymal transition                           |
| Eph        | erythropoietin-producing hepatocellular                        |
| EphA       | Eph receptor type-A  |
| EphB       | Eph receptor type-B  |
| ephrin     | Eph receptor-interacting (ligand)                              |
| ER         | estrogen receptor  |
| ERK        | extracellular signal regulated kinase                          |
| FGF        | fibroblast growth factor                                       |
| FGFR       | fibroblast growth factor receptor                              |
| FN         | fibronectin  |
| FRS        | fibroblast growth factor receptor substrate                    |
| GAG        | glycosaminoglycan  |
| GFP        | green fluorescent protein                                      |
| GPI        | glycosylphosphatidylinositol                                   |
| Grb        | growth factor receptor-bound                                   |
| HER        | Human Epidermal growth factor Receptor                         |
| HGF        | hepatocyte growth factor                                       |
| HSPG       | heparan sulphate proteoglycan                                  |
| Ig-like    | immunoglobulin-like  |
| JNK        | Janus kinase   |
| LTBP       | latent TGF- $\beta$ binding protein                            |
| LBD        | ligand-binding domain  |
| LOX        | lysyl oxidase  |
| LRP        | lipoprotein receptor related protein                           |
| MET        | mesenchymal-to-epithelial transition                           |
| MLC        | myosin light chain   |
| MMP        | matrix metalloproteinase                                       |
| mRNA       | messenger RNA  |
| MSC        | mesenchymal stem cell  |

|               |  |
|---------------|--|
| MT-MMP        | membrane type matrix metalloproteinase                                       |
| MT1-MMP       | membrane type-1 matrix metalloproteinase                                     |
| NCAM          | neural cell adhesion molecule  |
| N-cadherin    | neural cadherin  |
| IRAK          | interleukin-1 receptor-associated kinase                                     |
| PAI           | plasminogen activator inhibitor  |
| P-cadherin    | placental cadherin   |
| PDGF          | platelet-derived growth factor   |
| PDGFR         | platelet-derived growth factor receptor                                      |
| PDZ           | a post synaptic, disc large, and zona occludens protein domain-binding motif |
| PG            | proteoglycan   |
| PLC $\gamma$  | phospholipase C $\gamma$   |
| PMA           | phorbol 12-myristate 13-acetate  |
| PR            | progesterone receptor  |
| RECK          | reversion-inducing-cysteine rich protein with Kazal motifs                   |
| RTK           | receptor tyrosine kinase   |
| SAM           | a strile alpha motif   |
| SCID          | severe combined immunodeficiency   |
| SDF           | stromal cell-derived factor  |
| SFK           | Src family kinase  |
| SH2           | Src homology 2   |
| shRNA         | small-hairpin RNA  |
| siRNA         | small interfering RNA  |
| SNP           | single nucleotide polymorphism   |
| STAT          | signal transducer and activator of transcription                             |
| TGF- $\beta$  | transforming growth factor beta  |
| TGN           | trans-Golgi network  |
| TIMP          | tissue inhibitor of metalloproteinase  |
| TK            | tyrosine kinase  |
| TM            | trans-membrane domain  |
| TNF- $\alpha$ | tumour necrosis factor alpha   |
| uPA           | urokinase plasminogen activator  |
| VEGF          | vascular endothelial growth factor   |
| VEGFR         | vascular endothelial growth factor receptor                                  |
| vWF           | von Willebrand factor  |
| ZO-1          | zonula occludens 1   |



## ABSTRACT

Cancer metastasis is a stepwise process of cancer cell dissemination from a primary tumour into adjacent and distant tissues and causes around 90 % of cancer-associated mortality. Metastatic cancer dissemination is initiated and promoted by intracellular and intercellular signalling within tumour microenvironment. Extracellular matrix (ECM) degradation also promotes cancer cell invasion and metastasis in many types of cancer. Membrane type-1 matrix metalloproteinase (MT1-MMP) degrades variety of ECM components and cell surface proteins as well as modulates numerous intracellular signalling pathways to regulate cancer invasion. The molecular mechanisms of pro-invasive MT1-MMP activities are getting more attention to reveal cancer-associated cooperating signalling, which will aid in planning more efficient and effective therapeutic interventions for patients with cancer.

In the current studies, we performed a genome-wide gain-of-function human kinome screen to identify cancer-associated upstream and co-operating signalling for MT1-MMP activities. We identified both known and novel positive regulators of MT1-MMP. Among the novel MT1-MMP regulators we focused on the functions of two receptor tyrosine kinases, namely fibroblast growth factor receptor 4 (FGFR4) and Eph receptor type A2 (EphA2) in cancer cell invasion. Overexpression and aberrant signalling of these kinases are linked to aggressive cancer progression and anti-cancer drug resistance.

A single nucleotide polymorphism (SNP) of FGFR4 (G388R) associated with poor cancer prognosis was identified as a positive regulator of MT1-MMP activity. We revealed that the complexes of MT1-MMP and FGFR4-R388 risk variant stabilised and activated both MT1-MMP and FGFR4 proteins, resulting in enhancing FGF signalling and pericellular proteolytic activities of MT1-MMP. The FGFR4-R388-MT1-MMP axis induced epithelial-to-mesenchymal transition, promoting prostate carcinoma cell invasion and invasive growth within collagen matrix and in mouse xenograft models. In contrast, the FGFR4-G388 variant and MT1-MMP down-regulated each other.

EphA2 was co-expressed with MT1-MMP in invasive breast carcinoma cells, where EphA2 signalling increased *MT1-MMP* transcription. MT1-MMP in turn cleaved EphA2 in protein complexes on the same cell-surface. This cleavage coupled with EphA2-dependent Src activation triggered intracellular EphA2 translocation and an increase in RhoA activity, leading to actomyosine contraction, cell-cell repulsion, and cell junction disassembly. These signalling events ultimately induced cell invasion phenotype transition from collective to single-cell within three-dimensional collagen matrix and *in vivo*.

Taken together, these studies identified the FGFR4-R388 variant and EphA2 as novel co-operators for pro-invasive MT1-MMP activities in cancer invasion. FGFR4 genetic background affects the activity of an FGFR4-MT1-MMP complex in cancer progression, and an EphA2-MT1-MMP axis regulates cancer invasion plasticity. These findings provide novel insights into the cooperative molecular basis of pro-invasive capabilities of MT1-MMP and FGF and EphA2 signalling in cancer cell invasion.

# REVIEW OF THE LITERATURES

## INTRODUCTION

Human cancer develops over decades through a multi-step process. A normal cell transforms into a neoplastic state by accumulating a number of genetic changes and acquiring new properties; e.g. uncontrolled cell growth, evasion of apoptosis, and activation of cancer cell invasion and metastasis. Cancer cells can thus disseminate from a primary tumour throughout the body and form new tumours in distant tissues and organs. At the initial step of metastatic cancer progression, the activated cellular signalling mediates cytoskeletal dynamics in cancer cells and dissociation of cell-cell and cell-matrix junctions in a primary tumour, promoting local cancer cell invasion into adjacent tissues. Cancer cells further invade into blood and lymph vessels. Circulating cancer cells then exit these capillaries by infiltrating the underlying basement membranes, enter a new microenvironment, and ultimately form metastatic colonies. These events are driven by orchestrated processes including cancer cell motility, extracellular matrix (ECM) remodelling, and cell-cell and cell-matrix communications. Furthermore, during metastatic tumour progression cancer cells can also switch their invasive machineries to adapt to their surrounding environment, which is implicated in aggressive cancer metastasis and resistance to anti-cancer treatment.

In this work I have analysed the cooperative molecular mechanisms and cellular functions of pro-invasive MT1-MMP activities and FGF or Eph receptor tyrosine kinase signalling in cancer cells. I illustrate here the overview of cell-matrix and cell-cell communications within tumour microenvironment as well as cancer cell invasion plasticity. The review then focuses on human protein kinases. Specifically, I describe FGF and Eph receptor tyrosine kinase signalling in cancer. These signalling drive cancer progression or suppression in context-dependent manner. Finally, I emphasize MT1-MMP functions in cancer cell invasion and metastasis. The findings in this thesis study provide the novel insights into the molecular mechanisms of the FGFR4- or EphA2-MT1-MMP axes in cancer progression and different modes of cancer cell invasion. Understanding the molecular mechanisms of cancer progression and cancer cell invasion plasticity is likely to help to develop effective anti-invasion and anti-metastasis therapies.

## **1. Tumour microenvironment**

Activation of cancer cell invasion and metastasis is one of the hallmarks of cancer that assists in transforming a locally growing primary tumour into a systemic and life-threatening disease (Friedl and Alexander, 2011; Hanahan and Weinberg, 2011). This is not a single-cell process, but rather involves orchestrated multifaceted processes that include cancer cell-cell and cancer cell-stroma interactions in tumour microenvironment. The stroma consists of extracellular matrix (ECM), stromal cells, and various soluble factors including growth factors, chemokines, cytokines, and antibodies. Each stromal component can be associated with tumour progression.

### **1.1 Extracellular matrix (ECM)**

ECM provides structural support for multicellular architectures of tissues and organs (Hynes, 2009; Lu et al., 2012). ECM affects also fundamental cell functions, e.g. cell proliferation, differentiation, polarity, and migration and invasion through physical cell-ECM interactions and by modulating intracellular signalling via cell-surface receptors (Hynes, 2009; Lu et al., 2012). Structure and composition of ECM are constantly and dynamically remodelled, which is tightly controlled during developmental processes and in normal organ homeostasis. Impaired ECM dynamics is therefore implicated in many pathological conditions including cancer and tissue fibrosis (Lu et al., 2012). Aberrant ECM compromises its physical barrier and scaffolding functions, which can promote malignant transformation and progression through activation of oncogenic signalling pathways (Erler and Weaver, 2009; Lu et al., 2012). Furthermore, abnormal ECM can also influence stromal cell behaviour in tumour microenvironment, and thus facilitate tumour-promoting angiogenesis and inflammation.

#### **1.1.1 Structure of ECM**

The ECM is a complex assembly of many proteins composed of fibrillar and non-fibrillar collagens, other fibrillar proteins (e.g. fibronectin, elastin, and laminin), as well as glycosaminoglycans (GAG) and GAG-containing proteoglycans (PG). These tissue compartments form elaborate meshwork structures that can be classified into two separate entities based on the morphological and functional properties: interstitial matrix and basement membrane (BM).

##### **1.1.1.1 Interstitial matrix**

Interstitial matrix is a fibrillar three-dimensional (3D) meshwork structure (Egeblad et al., 2010; Lu et al., 2012). The components of this type of matrix are mainly synthesized by fibroblasts (Kisseleva and Brenner, 2008; Lu et al., 2012). Interstitial tissue is rich in fibrillar collagens, glycoproteins, as well as various GAGs and PGs. This type of matrix is thus highly charged and hydrated, which contributes to elastic and tensile strength of tissues (Egeblad et al., 2010b; Lu et al., 2012).

The main structural component of the interstitial ECM is type I collagen (Egeblad et al., 2010; Myllyharju and Kivirikko, 2001). Cross-linked meshwork structure of ECM composed of three  $\alpha$ -chain polypeptides of type I collagen which is predominantly catalysed by stromal

fibroblast-derived lysyl oxidase (LOX). The collagen triple-helix structure provides tissues with stable mechanical strength and high resistance to proteolytic degradation (Egeblad et al., 2010; Myllyharju and Kivirikko, 2001). Other major fibrillar proteins in this matrix are fibronectin and elastin (Hynes and Humphries, 1974; Magnusson and Mosher, 1998; Ruoslahti and Vaheri, 1974). Fibronectin is a glycoprotein that is abundant in most of ECM (Magnusson and Mosher, 1998). Through the interaction with other ECM components and cell surface adhesion molecules, e.g. integrins via Arg-Gly-Asp (RGD) sequence, fibronectin regulates ECM organization and cell-ECM adhesion (Magnusson and Mosher, 1998; Mao and Schwarzbauer, 2005). Fibronectin also facilitates collagen fibril organization by binding collagen (Velling et al., 2002). Elastin forms stable cross-linked fibres associated with microfibrils that are composed of fibrillins and other proteins, such as latent transforming growth factor- $\beta$  (TGF- $\beta$ ) binding proteins (LTBPs) (Hyytiainen et al., 2004). Elastic fibres form rubber-like polymers, which provide elastic stretch and recoil properties to special tissues such as blood vessels, lung, and skin (Kielty et al., 2002; Mithieux and Weiss, 2005).

The meshwork structure composed of the fibrillar proteins is associated with various GAGs and GAG-containing PGs. GAGs are long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain a sulphated sugar and an uronic acid such as glucuronate or iduronate. These sulphated GAGs attach covalently with core proteins and form PGs, such as aggrecan, decorin, and versican (Hardingham and Fosang, 1992; Kim et al., 2011). The only exception is hyaluronic acid which does not contain sulphate and does not bind proteins (Day and Sheehan, 2001). Hyaluronic acid is very hygroscopic, thus it is responsible for the gel-like character of tissues such as cartilage (Day and Sheehan, 2001). GAGs and PGs provide highly charged and aqueous environment surrounding cells, which allow rapid diffusion of small molecules such as salts, nutrients, and hormones (Hardingham and Fosang, 1992; Hynes, 2009; Kim et al., 2011). Importantly, PGs can act as reservoirs of growth factors, which may assist in the formation of gradients of the diffusible growth factor morphogens as well as accessibility and signalling direction of ligands to their cognate receptors (Hynes, 2009; Kim et al., 2011). For example, heparan sulphate proteoglycan (HSPG) acts as a co-factor for fibroblast growth factors (FGF) and its binding receptors (Hynes, 2009; Kim et al., 2011; Turner and Grose, 2010). Likewise, certain other growth factors such as a latent type TGF- $\beta$ , vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and Wnt also bind to pericellular PGs during signalling transduction (Hynes, 2009; Kim et al., 2011).

### **1.1.1.2 Basement membrane (BM)**

The BMs are specialized forms of thin dense sheet-like two-dimensional (2D) structures that are more compact and less porous than the interstitial matrix. The BMs underlie basolateral side to epithelial and endothelial cells, which supports apicobasal cellular polarity and tissue architecture (Kalluri, 2003). BMs also act as physical barriers, which separate the cells from stromal compartments (Kalluri, 2003). Unlike interstitial matrix, BMs consist mainly of network-forming type IV collagen, glycoprotein laminin, and linker proteins such as nidogen and perlecan (Kalluri, 2003; Rowe and Weiss, 2008). Laminin is a self-assembling protein, which is deposited to the basolateral side of the cell surface. The laminin network acts as a scaffold for further type IV collagen network formation and BM maturation (Kalluri, 2003).

Type IV collagen forms superstructures with highly cross-linked networks from six genetically different  $\alpha$ -chains (Kalluri, 2003). This laminin/ type IV collagen network is bridged by non-covalent interactions with linker glycoproteins, nidogens, and basement membrane-specific HSPG, perlecan. The whole network provides mechanical stability, selective filtration properties, and functions as a reservoir of growth factors for the BMs. Importantly, different isoforms of type IV collagen, laminin, and HSPG offer specific functions for the BMs with different tissue types and organs (Kalluri, 2003; Rowe and Weiss, 2008).

### **1.1.2 ECM Remodelling in Cancer**

In cellular microenvironment, the cells constantly modulate the structure and components of ECM by the activities of ECM remodelling enzymes, such as matrix metalloproteinases (MMP) and cross-linking enzyme, LOX (Barker et al., 2012; Cox and Erler, 2011; Kessenbrock et al., 2010; Lu et al., 2012). Reorganized ECM in turn influences adjacent cell functions and behaviour (Cox and Erler, 2011; Lu et al., 2012). Such cell-ECM bi-directional regulatory mechanism is tightly controlled, which maintains distinct tissue functions and organ homeostasis.

In pathological conditions, the architecture of matrix is severely altered by aberrant ECM degradation, deposition, and structure modification (Cox and Erler, 2011; Lu et al., 2012). BMs are often thinner and porous in solid tumours (Rowe and Weiss, 2008). Perturbing scaffolding and physical barrier functions of BMs can affect cell polarity and growth, and further promote cell invasion into adjacent interstitial matrix (Rowe and Weiss, 2008; Figure 1A). Breaching the BMs is mainly performed by MMPs (Hotary et al., 2006; Ota et al., 2009). For instance, membrane-anchored, membrane-type 1 (MT1-) MMP degrades the main components of BMs, such as type IV collagen and laminin (Giannelli et al., 1997; Hotary et al., 2006; Koshikawa et al., 2000; Ota et al., 2009). By cleaving BM components, MMPs generate biologically functional fragments, which can facilitate or inhibit cancer progression and invasion. For example, cleavage of laminin-5 by MT1-MMP and MMP2 generates pro-migratory  $\gamma 2$  subunit fragments, while fragments generated from type XVIII collagen by MMPs play as tumour suppressors (Giannelli et al., 1997; Koshikawa et al., 2000; Lin et al., 2001; Xu et al., 2001).

Interstitial collagens including type I and III collagens are in turn frequently accumulated and highly cross-linked in solid tumours, which increase tissue thickness and stiffness (Egeblad et al., 2010; Kauppila et al., 1998; Lopez-Novoa and Nieto, 2009; Zhu et al., 1995). The thickened ECM increases the bioavailability of various soluble growth factors and cytokines to their cognate cell-surface receptors and thus promotes further cancer progression and cancer-associated inflammatory responses (Egeblad et al., 2010; Margadant and Sonnenberg, 2010). The increased stiffness also enhances cell-ECM adhesion through mechano-transduced signalling by increased integrin clustering and focal adhesion assembly, leading to efficient cancer cell invasion (DuFort et al., 2011; Levental et al., 2009). Concomitantly, collagen fibres are often linearized and oriented in parallel to the adjacent tumours or in perpendicular to stroma (Provenzano et al., 2006; Figure 1B). This architecture is associated with enhanced cancer cell invasion, since cancer cells can use the radially aligned collagen

fibres as migration tracks (Provenzano et al., 2006). Cancer cells also modify pre-metastatic location for subsequent colonization by secreting ECM remodelling enzymes and recruiting cancer-associated stromal cells (Erler et al., 2009).

## **1.2 Communication between cancer cells and stromal cells**

During cancer progression, various types of stromal cells including fibroblasts, endothelial cells, and immune cells are recruited into a tumour. These cancer-associated stromal cells contribute to the tumour microenvironment formation together with cancer cells. Communication between cancer cells and these cells contributes to cancer progression (Hanahan and Coussens, 2012).

### **1.2.1 Cancer-associated fibroblasts (CAF)**

Fibroblasts are the main producers of ECM components (Rasanen and Vaheri, 2010). During developmental processes, fibroblasts actively assist in tissue morphogenesis, while in adults they are usually quiescent (Rasanen and Vaheri, 2010). Under wound healing and pathological conditions including cancer, fibroblasts are induced to be in an “activated” state (Orimo and Weinberg, 2006; Rasanen and Vaheri, 2010). Pre-existing fibroblasts within tumour environment are converted into an activated state by autocrine TGF- $\beta$  and stromal cell-derived factor-1 (SDF-1/ also known as CXCL12) signalling (Kojima et al., 2010). Alternatively, local mesenchymal stem cells (MSCs) and circulating bone marrow-derived MSCs are also recruited into a tumour by cancer cells (Direkze et al., 2004; Ishii et al., 2003). Such activated fibroblastic cells express  $\alpha$ -smooth muscle actin ( $\alpha$ -SMC). They are widely called “cancer-associated fibroblasts” (CAF; Rasanen and Vaheri, 2010; Figure 1B).

CAFs modulate components and structure of ECM within tumour microenvironment by expressing ECM components, growth factors, cytokines, and ECM remodelling enzymes (De Wever et al., 2008; Rasanen and Vaheri, 2010). By protease- and force-mediated matrix remodelling, CAFs degrade ECM and generate *de novo* gaps and microtracks that are used for cancer cell invasion by cohesive multicellular groups (Gaggioli et al., 2007; Scott et al., 2010; Zhang et al., 2006). CAFs also express numerous growth factors and cytokines, such as SDF-1, TGF- $\beta$ , and hepatocyte growth factor (HGF) and thus promote tumour growth and cancer cell invasion (Augsten et al., 2009; Bhowmick et al., 2004; De Wever et al., 2008; Orimo et al., 2005). Moreover, increased HGF and TGF- $\beta$  in the pericellular milieu may further accelerate autocrine CAF generation (Tyan et al., 2011; Wipff et al., 2007). The direct contact of cancer cells with fibroblasts can promote unimpeded cancer cell migration in interstitial matrix through membrane-bound ephrin ligand and Eph receptor signalling (Astin et al., 2010). CAFs also mediate cancer-promoting angiogenesis and macrophage recruitment by producing or releasing pro-angiogenic soluble factors or inflammatory cytokines (Calvo and Sahai, 2011; Hanahan and Coussens, 2012; Orimo and Weinberg, 2006; Rasanen and Vaheri, 2010).

### **1.2.2 Angiogenesis and lymphangiogenesis**

Along with increasing volume, a tumour requires nutrient, oxygen, and waste exchange (Hanahan and Weinberg, 2011). To supply these demands, tumours induce new sprouting of

endothelial cells from existing blood vessels, or recruit circulating bone marrow-derived endothelial progenitor cells (Bergers and Benjamin, 2003; Lyden et al., 2001; Purhonen et al., 2008). At the initial step of angiogenesis, cancer cells, CAFs, and immune cells within tumour microenvironment produce and release pro-angiogenic growth factors (e.g. VEGFs, FGF2, and SDF-1) that recruit endothelial cell sprouting through paracrine signalling (Baluk et al., 2005; Bergers and Benjamin, 2003; Hanahan and Coussens, 2012; Weis and Cheresh, 2011; Figure 1C). Sprouting endothelial tip cells require pericellular protease activities for degradation of endothelium BMs and interstitial matrix (Sounni et al., 2011; van Hinsbergh and Koolwijk, 2008). Besides ECM degradation, pericellular proteases are also involved in pro-angiogenic signalling activation (Sounni et al., 2011; van Hinsbergh and Koolwijk, 2008). For example, MT1-MMP processes LTBP on endothelial cells and releases pro-angiogenic TGF- $\beta$  (Tatti et al., 2008). This protease also generates biologically functional fragments by cleavage of thrombospondin-1 (TSP-1) and nidogen-1, which promotes neovascular development (Koziol et al., 2012). Unlike normal blood vessels, tumour-associated vessels have poor basement membrane deposition and loose perivascular cell association with endothelial cells, resulting in porous and leaky blood vessels with abnormal blood flow (Baluk et al., 2005; Bergers and Benjamin, 2003).

### **1.2.2.1 Cancer cell metastasis through circulation**

Fluid, proteins, and cells that leak out from the blood vessels are taken up by neighbouring lymphatic vessels via overlapping endothelial flaps (Norrmen et al., 2011). Lymphatic vessels are essential for transportation of immune cells (Tammela and Alitalo, 2010). Furthermore, together with blood vascular system, lymphatic capillaries are used as main routes for metastasizing cancer cell (Tammela and Alitalo, 2010; Weis and Cheresh, 2011; Figure 1D). Compared with blood vessels, lymphatic vessels have wider diameters as well as more porous and permeable walls (Norrmen et al., 2011; Tammela and Alitalo, 2010). These structural differences can influence metastatic cancer cell dissemination through blood or lymphatic vessels. For example, cohesive breast cancer cell groups enter only into lymphatic vessels, whereas singly invading cells after activation of TGF- $\beta$  signalling can move into both blood and lymphatic capillaries, ultimately leading to blood-borne metastasis *in vivo* (Giampieri et al., 2009). Therefore, cancer cell metastasis into regional lymph nodes through lymphatic vessels is the first important step for cancer cell metastasis (Tammela and Alitalo, 2010). VEGF-C and VEGF-D produced by cancer cells and immune cells are the main pro-lymphangiogenic factors that also promote cancer cell entry into lymphatic vessels and regional lymph nodes (Tammela and Alitalo, 2010).

Direct contacts between cancer cells and endothelial cells through cell surface receptors regulate intra- and extravasation of cancer cells (Mierke, 2008). For extravasation of cancer cells from the circulation, circulating cancer cells interact with endothelium and underlying BMs and then degrade these barriers (Kargozaran et al., 2007; Reymond et al., 2012). Perivascular cancer-associated immune cells also assist in cancer cell intravasation (Wyckoff et al., 2007). Only a limited population of cancer cells achieves metastatic colonization due to the poor interaction of cancer cells and the endothelium in circulation and organ-specific barriers that block cancer cell extravasation (Nguyen et al., 2009).

### 1.2.3 Infiltrating immune cells

Massive infiltration of immune cells including macrophages, neutrophils, T-cells and other leukocytes into tumours are frequently observed (Grivennikov et al., 2010; Mantovani et al., 2008). The relationship of immune systems with cancer development is complex. In the early stage of tumour, immune cells inhibit tumour growth by recognition and rejection of cancer cells (Grivennikov et al., 2010; Mantovani et al., 2008). Cancer cells, in turn, can also manipulate certain immune cells to tumour promoting phenotypes by producing various cytokines and chemokines within tumour microenvironment (Hanahan and Weinberg, 2011; Joyce and Pollard, 2009; Mantovani et al., 2008). Cancer-associated leukocytes then obtain tumour-promoting functions whereby they can promote angiogenesis as well as cancer cell invasion, intravasation and ultimately metastasis (Hanahan and Coussens, 2012; Joyce and Pollard, 2009; Mantovani et al., 2008; Figure 1E).

These cascades of communication between cancer cells and stromal cells will aid in generation of a tumour microenvironment that further facilitates cancer progression.

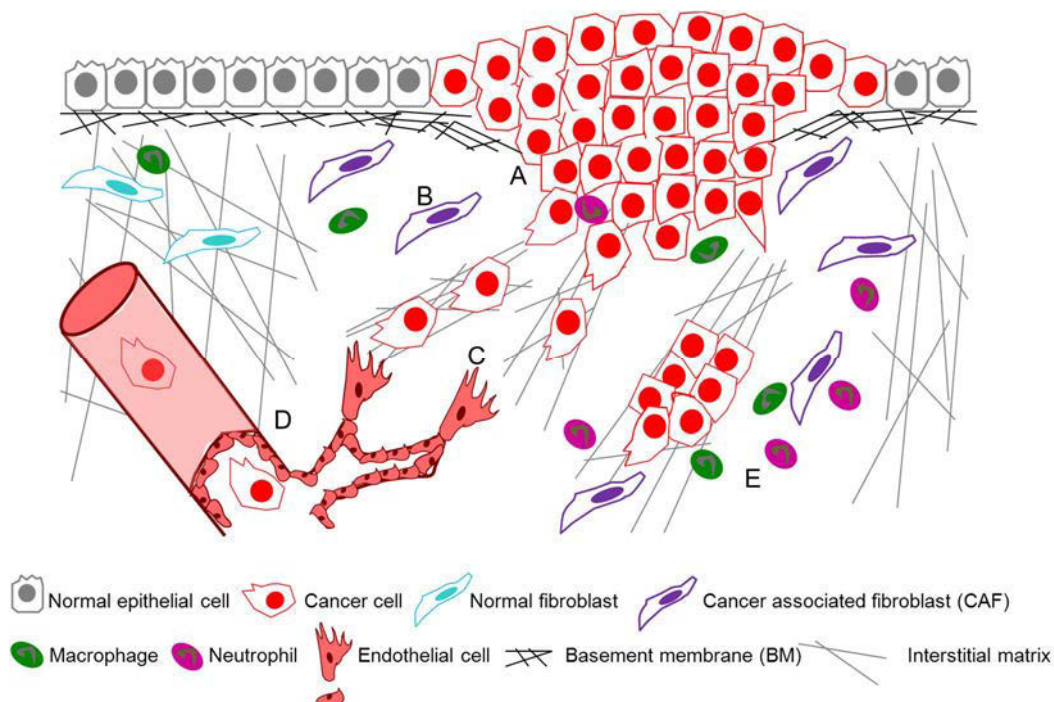


Figure 1. Communications of cancer cells and stroma in tumour microenvironment. A) At the initial step of cancer invasion, cancer cells infiltrate into adjacent interstitial compartment by degrading BMs and interstitial matrix by ECM remodelling enzyme activities. B) Cancer cells also generate cancer-associated fibroblasts (CAFs), which contribute to abnormal ECM remodelling, e.g. ECM degradation and linearized interstitial collagen fibre formation. The linearized collagen fibres are oriented in perpendicular to stroma and used as a “highway” for invading cancer cells. C) During tumour growth, cancer cells induce cancer-associated new blood and lymphatic vessel formation. D) Leaky tumour-associated vasculatures and lymphatic capillaries are used as routes for cancer cell dissemination to distant sites. E) In the late stage of tumour, immune cells are recruited to the tumour to promote cancer progression. (Adapted and modified from Lu et al., 2012).



## 2 Cancer cell invasion

Cancer cell invasion from a primary tumour into adjacent interstitial compartment occurs by cohesive cell groups and/or as single cells (Friedl and Alexander, 2011). Different modes of cancer invasion rely on the cell type, cell-autonomous molecular mechanism, and cancer cell-matrix communications within tumour microenvironment (Friedl and Alexander, 2011).

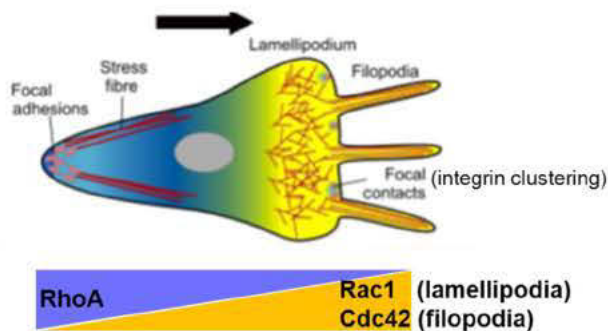


Figure 2. RhoGTPase activation during cell migration on 2D culture. Black arrow indicates the direction of cell migration. (Adapted and modified from Mayor and Carmona-Fontaine, 2010).

al., 2003; Figure 2). The front-rear polarity of migrating cells is controlled by Rho-GTPase family-mediated actin cytoskeleton dynamics and integrin-mediated cell-matrix adhesion (Ridley et al., 2003; Figure 2). Rho-GTPases, Rac1 and Cdc42 drive actin assembly to form lamellipodia or filopodia that engage with underlying substrate via integrins at focal adhesion sites, while RhoA activation induces stress fibre formation through actomyosin contraction (Ridley et al., 2003; Figure 2).

Unlike rigid and non-elastic 2D culture, cells in 3D matrices and *in vivo* display greater range of protrusive structures and invasion modalities (Friedl and Alexander, 2011; Sanz-Moreno and Marshall, 2010). The different invasion modes are interchangeable and they are driven by distinct cell-cell communications and in response to changes in surrounding ECM characters such as rigidity, deformability, density, and pore size (Friedl and Alexander, 2011; Sanz-Moreno and Marshall, 2010). Pericellular ECM proteolytic activities are also involved (Harunaga and Yamada, 2011; Sabeh et al., 2009).

### 2.1 Single-cell invasion

Single-cell invasion in 3D models has been analysed using leukocytes, neural crest cells, and *Dictyostelium discoideum* amoeba, which provide important insights into cellular and molecular mechanisms of different modes of single-cell invasion (Friedl et al., 2001; Mayor and Carmona-Fontaine, 2010; Ridley et al., 2003). For example, leukocytes are constitutively migrating as rounded-shape individual cells, whereas neural crest cells display rather elongated mesenchymal-type phenotype (Friedl et al., 2001; Madsen and Sahai, 2010; Mayor and Carmona-Fontaine, 2010). In addition, migrating leukocytes use a similar mechanism with amoeba that squeezes through small spaces between ECM by rapid changes in cell shape

and direction (Friedl et al., 2001; Lammermann and Sixt, 2009; Wolf et al., 2003). Of note non-cancer cell migration is tightly controlled. Although cancer cells use similar mechanism for cell invasion as observed with non-cancer cells, they lost cell-cell contact-mediated immobilizing signalling and thus invade aggressively (Huttenlocher et al., 1998).

### 2.1.1 Amoeboid-type invasion

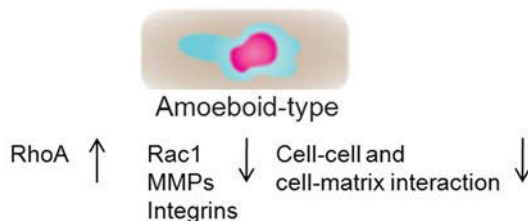


Figure 3. Characters of an amoeboid-type single-cell invasion. (Adapted and modified from Friedl and Alexander, 2011 and Friedl et al., 2012)

Similar to amoeba and leukocyte migration, cancer cells display rounded sphere-like morphology in combination with low adhesion toward underlying matrix (Lammermann and Sixt, 2009). These cells invade at relatively high velocities (2-25  $\mu\text{m}/\text{min}$ ) coupled with constant formation and retraction of blebs or other types of smooth membrane protrusions (e.g. pseudopodia) and change the cell shape ( Brabek et al., 2010; Lorentzen et al., 2011; Paluch et al., 2006; Poincloux et al., 2011). RhoA-ROCK and Cdc42-Pak1-mediated actomyosin

contractility drive this invasion mode, while activated Rac1 polarization at the leading edge is not required (Calvo et al., 2011; Friedl and Alexander, 2011; Sanz-Moreno and Marshall, 2010; Figure 3). Amoeboid-type cells apt to invade in absence of integrin-mediated cell-ECM adhesion and MMP-dependent ECM remodelling (Wolf et al., 2003; Figure 3). The cells rather squeeze and intercalate between pre-existing gaps and trails within ECM matrix (Wolf et al., 2003).

In addition to leukemias and lymphomas, amoeboid-type invading cells are also observed in subgroups of many types of carcinomas, such as breast, prostate, and small-cell lung carcinomas, as well as melanoma (Madsen and Sahai, 2010; Sanz-Moreno and Marshall, 2010; Wolf et al., 2003).

### 2.1.2 Mesenchymal-type invasion

Cancer cells can also invade as single-cells displaying elongated, spindle-shaped fibroblast-like phenotype (Friedl and Alexander, 2011). The mesenchymal-type cell invasion is slower than amoeboid-type invasion (0.1-2  $\mu\text{m}/\text{min}$ ) driven by Rac1-mediated actin polymerization coupled with integrin- $\beta 1$  and  $\beta 3$ -mediated cell-matrix adhesion as well as protease-dependent ECM remodelling (Friedl and Wolf, 2004; Sanz-Moreno and Marshall, 2010).

The migration processes of mesenchymal-type invasion includes five separate steps as shown in Figure 4 (Friedl and Wolf, 2009; Friedl and Alexander, 2011). 1) Chemokine gradient or growth factor signalling initiate to polarize activated Rac1 and Cdc42 at the membrane ruffles, leading to actin stress fibre polymerization and formation of membrane protrusion (e.g. lamellipodia and filopodia) (Sanz-Moreno and Marshall, 2010). 2) The membrane protrusions adhere toward ECM through clustered integrins at focal adhesion sites that connect extracellular matrix to intracellular cytoskeleton. 3) MMPs degrade subcellular ECM followed by mechanical force to push connected ECM, generating a gap and migration track between matrixes. 4) An increased RhoA activation in central to rear part of migration cells

induces actomyosin contraction. 5) An increased cell body contraction induces retraction of the rear tail of migrating cells, inducing moving forward by sliding the cell body and nuclear. Such translocation of cell body can further promote cell migration (Friedl and Wolf, 2009; Friedl and Alexander, 2011).

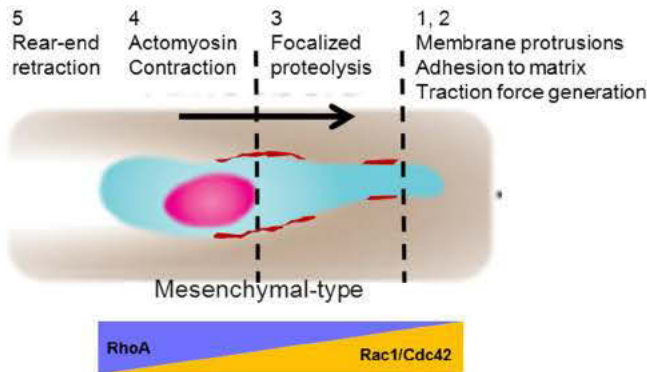


Figure 4. Five steps of mesenchymal-type cell invasion. Black arrow indicates direction of cell migration and red indicate pericellular proteolysis. (Adapted and modified from Friedl and Alexander, 2011 and Friedl et al., 2012).

Cells migrating in the mesenchymal-type cell manner can be observed in many types of cancer including breast, prostate, colon, and melanomas. These cells have undergone EMT whereby epithelial-type cohesive cells lose E-cadherin-based cell-cell contacts and migrate by the mesenchymal-type elongated single-cell phenotype (Madsen and Sahai, 2010; Thiery et al., 2009).

## 2.2 Multicellular invasion

Multicellular invasion is largely observed in sprout formation of blood and lymph capillaries, branching morphogenesis (e.g. mammary and prostate glands), neural crest movement, and wound healing during developmental processes and in adult tissue homeostasis (Friedl and Gilmour, 2009). In cancer, many types of carcinoma cells also invade as cohesive cell cohorts from the same primary tumours (Friedl et al., 2012). In contrast to single-cell invasion, collectively migrating cells maintain connections between neighbouring cells through cell adhesion molecules, such as cadherins and immunoglobulin superfamily members (Friedl and Gilmour, 2009; Friedl et al., 2012). Multicellular invasion also displays different types of invasion, such as multicellular streaming and collective invasion with cluster, tube and strand phenotypes (Friedl and Gilmour, 2009; Friedl et al., 2012; Mayor and Carmona-Fontaine, 2010).

### 2.2.1 Multicellular streaming

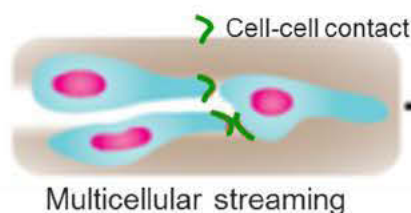


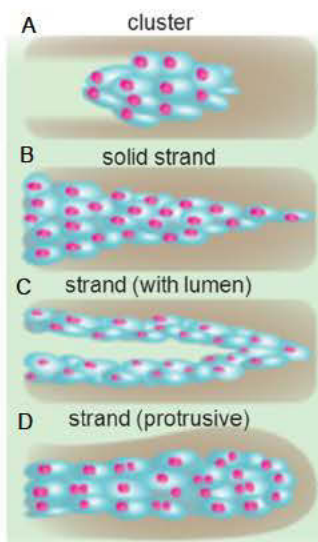
Figure 5. Multicellular streaming. The cell-cell contact is mediated by pro-migratory cadherins, e.g. N-cadherin and cadherin-11. (Adapted and modified from Friedl and Alexander, 2011 and Friedl et al., 2012)

When individual cells jointly attract to chemokine or morphogen gradient, the mesenchymal-type cells invade one after each other using same migration tracks within the tissues (Figure 5). This style of cell invasion is frequently observed in neural crest cell migration (Theveneau and Mayor, 2012b). The migrating cells in a group retain independent cytoskeleton dynamics including actomyosin-mediated retraction of their rear tails, while their cell-cell contacts are maintained

transiently and weakly by pro-migratory cadherins, such as neural (N)-cadherin and cadherin-11 (Mayor and Carmona-Fontaine, 2010; Theveneau and Mayor, 2012a; Figure 5). These cadherins can also activate intracellular signals involved in cell polarity and cytoskeleton organization (Carmona-Fontaine et al., 2011; Theveneau et al., 2010). Multicellular streaming movement is coordinated by repulsive cell movement upon cell collision mediated by RhoA activation (contact inhibition of locomotion; CIL) and Rac1-mediated cell polarization toward chemoattractant (co-attraction; CoA); Theveneau and Mayor, 2013). The capacities of transient cell-cell contact and independent actin cytoskeleton dynamics of each cell allow cells to migrate rapidly toward the chemoattractant as a multicellular group (Theveneau and Mayor, 2013).

Multicellular stream invasion is observed in orthotopic breast cancer, where a paracrine loop between cancer cells and macrophages promotes swarm-like streaming motility and intravasation of cancer cells (Roussos et al., 2011).

### 2.2.2 Collective cell invasion



Collective invasion

Figure 6. Phenotypes of collective cell invasion. (Adapted and modified from Friedl and Alexander, 2011 and Friedl et al., 2012)

Invading cells form cohesive multicellular group with constitutive cell-cell contacts. Similar to singly invading cells, the leader cells located at the invasive front form membrane protrusions, connect to surrounding substrates through  $\beta$ 1- and  $\beta$ 3-integrins in focal adhesion sites, and generate a migration trail by local ECM proteolysis. The following cells use and widen this migration path by degrading and pushing the attached walls (Wolf et al., 2007). Different from the single-cell invasion and multicellular streaming, collectively invading cells do not retract the rear tail of each cell, and rather pull neighbouring cells. In many cases, the following cells retain epithelial cell morphology coupled with cell-cell contacts through tight and adherent junctions (Friedl et al., 2012). The cell cohort therefore is maintained, which also control a position and polarity of each cell within the collective structure (Hidalgo-Carcedo et al., 2011). Ultimately, collective invading cells behave as a “mega-cell” with synchronized cytoskeletal dynamics (Friedl and Alexander, 2011).

Collective cancer cell invasion is frequently observed in the interface between tumour and stroma where cancer cells adopt different morphologies depending on the cell type, ECM structure, and cell-cell and cell-ECM communications (Friedl and Gilmour, 2009; Friedl et al., 2012). For examples, in prostate, breast, and pancreas carcinomas, the cells invade as small clusters or strands with one or several leader cells that drive forward migration by forming membrane protrusions and pericellular proteolysis (Friedl et al., 2012; Wolf et al., 2007; Figure 6A-C). The leader cells are followed by cohesive cell group (cluster and strand) and even more polarized followers may form an inner luminal structure (strand with lumen) (Friedl et al., 2012; Wolf et al., 2007; Figure 6A-C). CAFs can also act as leader cells for

collectively invading squamous carcinoma cells that retain epithelial characters (Chaudhry et al., 2013; Gaggioli et al., 2007). In addition, cells also invade in a manner dependent on placental (P)-cadherin-mediated cell-cell contacts, where cells in a group do not have specific leader cells and constitutively change their location (protrusive strand; Ewald et al., 2008; Gray et al., 2010; Nguyen-Ngoc et al., 2012; Figure 6D). The cells may rather use mechanical force to push surrounding ECM and to generate space for invasion (Ewald et al., 2008; Gray et al., 2010). To date, this type of collective invasion has been observed only during developmental processes including mammary branching (Ewald et al., 2008; Nguyen-Ngoc et al., 2012). However, the similar invasive mechanism would be predicted to be observed in pathological conditions.

## 2.3 Plasticity of cancer cell invasion

During metastatic cancer progression, invading cancer cells encounter different tissue microenvironment, consisting of a variety of ECM components, stromal cells, and soluble growth factors and cytokines. In addition, cancer therapy challenges, e.g. irradiation, chemotherapy, and surgery, can also contribute to microenvironment stress. To adapt to diverse microenvironmental conditions, cancer cells modulate their invasion modes by intracellular signalling through the cell surface receptors and cell-cell and cell-matrix interactions as well as by the physical properties of ECM (De Bock et al., 2011; Kargiotis et al., 2010; Friedl and Alexander, 2011; Wolf and Friedl, 2009). Cancer invasion is thus regarded as an adaptive and plastic process, which can facilitate cancer cell metastasis and further contribute to resistance to anticancer therapy (Alexander and Friedl, 2012).

### 2.3.1 Collective-to-single cell transition

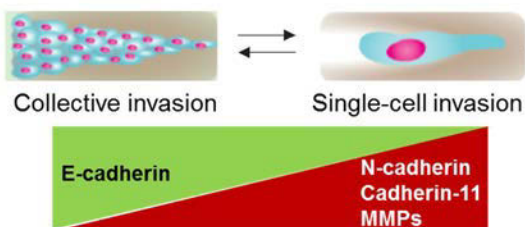


Figure 7. Collective-to-single cell transition (Adapted and modified from Friedl and Alexander, 2011 and Friedl et al., 2012)

Collectively invading cancer cells can transit to individual phenotype in a tumour. For example, multicellular cohesive groups loose tight cell-cell contacts by down-regulation of cell-cell adhesion molecules, ultimately resulting in individual cell dissemination from multicellular groups (collective-to-single cell transition; Figure 7). Conversely, if singly moving cells up-regulate cell-cell adhesion molecules, the cells start to aggregate and move as cohesive multicellular groups (single-to-collective transition; Friedl and Alexander, 2011; Thiery et al., 2009; Figure 7). EMT and its reversible process mesenchymal-to-epithelial transition (MET) are involved in these processes.

#### 2.3.1.1 Epithelial-to-mesenchymal transition (EMT)

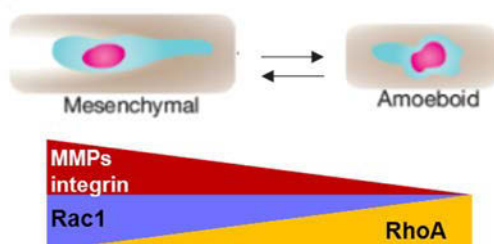
During embryonic development and morphogenesis as well as in pathological conditions including cancer, epithelial cells can acquire mesenchymal capabilities, resulting in loss of tight contacts with their neighbours and apicobasal cellular polarity concomitant with gain of migratory and invasive capacities (Thiery et al., 2009). This is an important initial step for local cancer invasion. In addition to the motile property, cancer cells undergoing EMT gain

anti-apoptotic and anti-senescence properties, and further stem cell-like characteristics (Thiery et al., 2009).

In tumour microenvironment, many growth factors including FGF, HGF, EGF, TGF- $\beta$ , and Wnt in stroma induce EMT of primary tumour cells, which can locally facilitate cell escape from a primary tumour into the adjacent ECM as multicellular groups or as single cells (Friedl et al., 2012; Polyak and Weinberg, 2009). In addition, hypoxic conditions, frequently existing in tumour microenvironment, also promote EMT by increasing the expression of c-Met/HGF receptor in cancer cells that enhances HGF-induced cancer cell migration and dissemination (Pennacchietti et al., 2003). These signalling pathways activate one or several transcriptional repressors including ZEB1, Twist, and Snail 1 and 2, which inhibit *E-cadherin* transcription (Peinado et al., 2007; Vandewalle et al., 2009). Concomitantly, these E-cadherin repressors, e.g. Snails and Twist, can induce expression of mesenchymal phenotype-associated cadherins, e.g. N-cadherin and cadherin-11, resulting in weakened cell-cell adhesion and disturbance of apicobasal cellular polarity (Peinado et al., 2007; Vandewalle et al., 2009). The downregulation or loss of E-cadherin coupled with up-regulation of N-cadherin and/or cadherin-11 is known as “cadherin-switch”, which is one of the hallmarks of EMT to allow the cells to acquire motile mesenchymal-type spindle phenotype (Thiery et al., 2009; Yilmaz and Christofori, 2010).

The cells undergoing EMT can become tip cells located at the leading edge of cohesive multiple cell groups (Wolf et al., 2007). The cells further loosen cell-cell junctions; they can invade by multicellular streaming and/or mesenchymal-type individual cells. Furthermore, the cells acquire stem cell-like traits to disseminate to distant metastases as undifferentiated single cells (Polyak and Weinberg, 2009; Theveneau and Mayor, 2013; Thiery et al., 2009; Yilmaz and Christofori, 2010). Mesenchymal-type cells are characterized by cadherin-switch and upregulation of *vimentin* and *MMPs* including MMP1, MMP9, MT1-MMP and MT2-MMP (Miyoshi et al., 2004; Tao et al., 2011; Vandewalle et al., 2009). These MMPs cleave E-cadherin and thus further facilitate cell-cell dissociation. Importantly, EMT is a reversible process that is transiently controlled in the local microenvironment. At the reached new microenvironment, the disseminated mesenchymal-type or stem cell-like undifferentiated cells from the primary tumours can revert to differentiated epithelial-like phenotype (mesenchymal-to-epithelial transition; MET) and ultimately form metastatic colonies, where EMT-inducible signalling are not activated (Polyak and Weinberg, 2009; Thiery et al., 2009; Yilmaz and Christofori, 2010).

### 2.3.2 Mesenchymal-to-amoeboid transition



Singly invading cells can flexibly interchange between mesenchymal and rounded amoeboid-type phenotypes in different stages of metastatic process and therapeutic challenge (Alexander and Friedl, 2012; Friedl and Alexander, 2011; Sanz-Moreno and Marshall, 2010; Figure 8).

Figure 8. Mesenchymal-to-amoeboid transition. (Adapted and modified from Friedl and Alexander, 2011)

Mesenchymal-to-amoeboid transition is regulated by the balance between Rac and RhoA activity (Sanz-Moreno and Marshall, 2010; Figure 8). For example, mesenchymal-type cells can switch to amoeboid-type cells by inhibition of Rac activity concomitant with activation of Rho/ROCK-mediated actomyosin contractility (Sahai and Marshall, 2003; Sanz-Moreno and Marshall, 2010). Rho-ROCK axis is regulated by multiple pathways including signalling through EphA2 (Parri et al., 2009; Taddei et al., 2011), inhibition of negative Rho regulators (e.g., p90RhoGAP; Nimnual et al., 2003), and inhibition of chemokine-mediated Rac activation (Gerard et al., 2007; Sanz-Moreno et al., 2008). Interestingly gene mutations, e.g. loss or mutation of tumour suppressor p53, can also promote rounded amoeboid-type cell dissemination through RhoA-ROCK signalling activation, promoting tumour cell invasion and metastasis (Gadea et al., 2007).

ECM remodelling is also associated with mesenchymal-to-amoeboid transition. The size of pore present in BMs and interstitial tissues *in vivo* is less than 1  $\mu\text{m}$  and 2-30 $\mu\text{m}$  diameter, respectively (Weigelin et al., 2012; Wolf et al., 2009). The largest and most rigid organelle in a cell is nucleus that has around 50-100  $\mu\text{m}^2$  cross-sectional areas (Wolf et al., 2013). Mesenchymal-type invading cells adhere to ECM and cleave collagen fibrils by MMPs to widen the available spaces for invading cell body in tissues (Wolf et al., 2007; Sabeh et al., 2009), while cancer cells can move through  $>7 \mu\text{m}^2$  space by deforming cell body and nucleus without proteolytic activity (Wolf et al., 2013). Therefore, in looser interstitial tissues inhibition of MMP activities can switch the cancer cell invasion mode from mesenchymal-type to protease-independent amoeboid-type movement (Wolf et al., 2003).

### 3 Human protein kinases/Human kinome

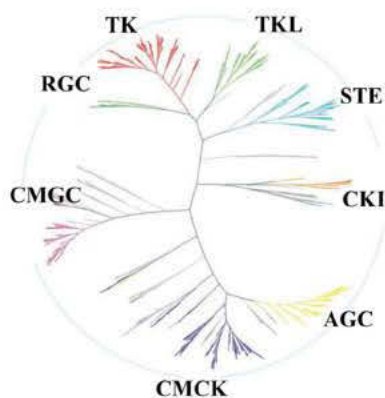


Figure 9. Human kinome (Adapted and modified from Manning et al., 2002)

Cells adapt to surrounding microenvironment by responding to signals from surrounding ECM and neighbouring cells, leading to biological responses and many cellular processes (Manning et al., 2002). Protein kinases are central players in cell signalling through modification of substrate activity that control fundamental cell-cell and cell-matrix communications, as well as cellular functions, e.g. cell proliferation, differentiation, metabolism, apoptosis, cell-cycle, cytoskeletal rearrangement, and cell motility (Manning et al., 2002). Thus, mutations and aberrant regulation of protein kinases are closely implicated in human disease.

The human genome encodes approximately 518 protein kinase genes (Manning et al., 2002). As shown in Figure 9, these kinases have been identified and categorized into 10 broad groups: AGC (containing protein kinase A, G, and C); CAMK (calcium/calmodulin-dependent protein kinase); CMGC (containing cyclin-dependent kinase, mitogen-activated protein kinase, glycogen synthase kinase 3, and CDC2-like); RGC (receptor guanylate cyclase); TK (tyrosine kinase); TKL (tyrosine kinase-like); STE (homologs of yeast sterile 7, 11, and 20); CK1 (casein kinase 1); atypical; and “other” (Manning et al., 2002).

### 3.1 Protein kinases in cancer

Cancer cells accumulate somatic alterations, such as gene amplification, deletion, translocation, or point mutations; however, most mutations are not directly involved in oncogenesis (“passenger mutation”). Kinases bearing “driver mutation” confer survival and growth advantages on cancer cells and thus promotes cancer progression (Torkamani and Schork, 2009; Witsch et al., 2010). Many driver mutations in protein kinase genes are associated with progression of certain cancer, e.g. human epidermal growth factor receptor 2 (HER2/erbB2) amplification in approximately 25 % of breast carcinomas (Rexer and Arteaga, 2012), v-raf murine sarcoma viral oncogene homolog B1 (B-RAF) mutation in 50-60 % of advanced melanomas (Ribas and Flaherty, 2011), and EGFR mutation in 10-20 % of non-small-cell lung carcinomas (Pao and Chmielecki, 2010). Furthermore, recent cancer genome sequencing studies have identified over 100 potential cancer driver mutations (Figure 10A; Greenman et al., 2007; Kan et al., 2010; Zhang and Daly, 2012). Cancer-specific aberrant expression and activation levels of kinases as well as cross-talk with other kinase signalling pathways can be studied by mass spectrometry-based proteomic approaches (Kolch and Pitt, 2010). For example, the proteomic approach found the prominent Src family kinase (SFK) signalling activation and its cooperative pro-invasive and -proliferative TK signalling network in invasive breast cancer cells (Hochgrafe et al., 2010).

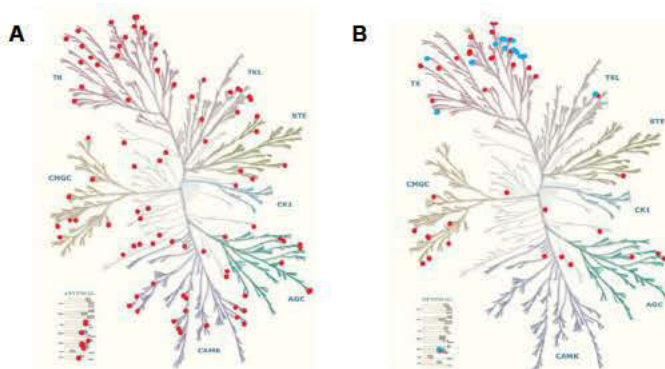


Figure 10. The untapped potential of the human kinome. A) Kinases that are mutated in common human cancers. The top 100 kinases ordered by conditional probability of carrying at least one driver mutation are highlighted (Greenman et al., 2007). B) Human protein kinases targeted by inhibitors that are currently in clinical trial in cancer patients. Targeted kinases are highlighted by red and blue circles (blue: FDA-approved therapies). (Adapted and modified from Zang and Daly, 2012)

Based on these studies, preclinical trials of targeting oncogenic kinases or cancer-specific kinase signalling networks have been launched using small-molecule kinase inhibitors or monoclonal antibodies (Dar and Shokat, 2011; Li and Zhu, 2010; Lofblom et al., 2011; Zhang et al., 2009). However, a relatively small number of kinases are currently evaluated and targeted in clinical trials (Figure 10B, red and blue dots indicate targeted kinases in clinical trials. Blue dots indicate FDA-approved drugs; Zhang and Daly, 2012). Although some molecular targeted therapies have had clinical success, clinical trials face with big challenge to drug resistance by genetic and/or epigenetic kinase mutations, or

activation of alternative signalling pathways (Zhang et al., 2009). Therefore, effective target therapies require detailed understanding of the molecular mechanisms and functions of the targeted kinases, the impact of the therapy on intracellular signalling, and drug resistance mechanism. Moreover, identification of cancer-specific gene alterations in kinases may help design personalized cancer therapy and stratification of cancer patients (Zhang and Daly, 2012).



## 4 Receptor tyrosine kinases (RTKs) in cancer

Receptor tyrosine kinases (RTK) are cell-surface receptors that have an intracellular tyrosine kinase domain. At cell surface, RTKs play critical roles in cell-cell and cell-ECM communications through binding to external signals and transmitting them into intracellular signalling cascades (Lemmon and Schlessinger, 2010). They regulate many of biological responses, such as cell proliferation, differentiation, survival, cell-cycle, intercellular communication and cell migration (Lemmon and Schlessinger, 2010). Human RTK is comprised of around 60 members that have been divided into 20 subfamilies. They share common structures with ligand-binding extracellular domain, a single-pass transmembrane domain, and cytoplasmic region that contain a protein kinase domain. Upon ligand binding, RTKs become catalytically active through phosphorylation of tyrosine residues and conformational changes within kinase domain. The activated RTKs then recruit signalling adaptor proteins to initiate multiple downstream signalling cascades (Lemmon and Schlessinger, 2010). The strength and retention of RTK signalling are regulated by ligand-bound RTK internalization and endocytic trafficking (Parachoniak and Park, 2012). The internalized RTKs are transported into nucleus, recycling endosomes, or lysosomes or proteasomes where RTKs are degraded and thus RTK signal is terminated (Parachoniak and Park, 2012).

Increased amount of evidence indicates that aberrant RTK signalling is implicated in cancer progression and cancer therapy resistance (Lemmon and Schlessinger, 2010; Parachoniak and Park, 2012; Witsch et al., 2010; Zhang et al., 2009). Indeed, many driver mutations have been found in different types of cancer (Lemmon and Schlessinger, 2010; Witsch et al., 2010). Deregulated and dysfunctional RTK signalling are frequently caused by gain-of-function mutation, SNP, RTK gene amplification, chromosomal translocation, and aberrant autocrine and paracrine signalling (Lemmon and Schlessinger, 2010; Witsch et al., 2010).

### 4.1 Fibroblast growth factor receptor family

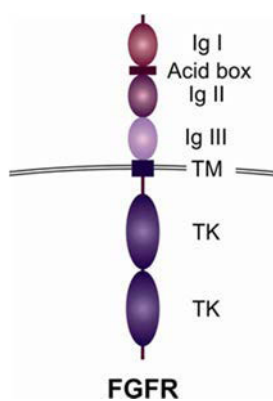


Figure 11. Structure of FGFR.

Ig, immunoglobulin-like domain; TM, transmembrane domain; TK, tyrosine kinase domain (Adapted and modified from Turner and Grose 2010)

The mammalian FGFR family comprises four members, FGFR1, FGFR2, FGFR3, and FGFR4, as well as one additional receptor, FGFR5 (FGFRL1) (Turner and Grose, 2010). FGFR5 does not contain a kinase domain but is able to bind the ligands and may act as a negative regulator of FGFR signalling (Wiedemann and Trueb, 2000). FGFR1-4 have highly conserved structure. It consists of an extracellular region containing three immunoglobulin-like (Ig-like) domains, a single transmembrane domain (TM) and an intracellular TK domain (Turner and Grose, 2010; Figure 11). The second and third Ig-like domains can bind the ligands, FGFs that consist of 18 members (FGFs1-10, 16-23) (Turner and Grose, 2010). FGFs are secreted glycoproteins that are sequestered to ECM and cell surface by binding to HPSGs (Turner and Grose,

2010). HPSGs can also facilitate FGF-FGFR dimerization by simultaneously binding to both FGF and FGFR on the cell surface (Mohammadi et al., 2005; Turner and Grose, 2010). The specificity of FGF-FGFR interaction relies on alternative splicing variants of the receptors and tissue-specific expression pattern of FGF, FGFR, and HPSGs (Eswarakumar et al., 2005; Turner and Grose, 2010). Two splice variants in the Ig-III domain of FGFR1-3 provide b (FGFR1-3IIIb) and c (FGFR1-3IIIc) isoforms. The IIIb and IIIc isoforms are predominantly expressed in epithelial and mesenchymal cells respectively, and they display distinct FGF binding capacities (Beenken and Mohammadi, 2009). Each FGF binds to either epithelial or mesenchymal FGFRs, with the exception of FGF1 which activates both splice isoforms (Eswarakumar et al., 2005).

#### 4.1.1 FGF signalling

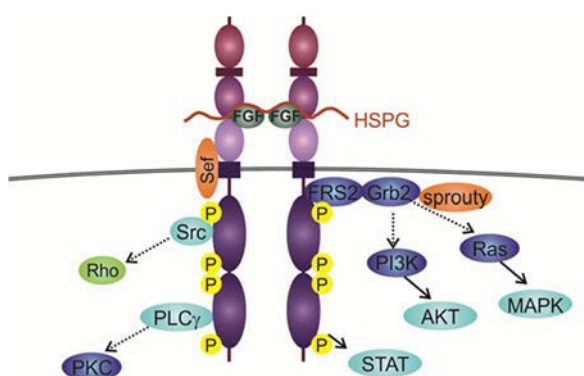


Figure 12. FGFR signalling network.

Upon ligand binding, the receptors are dimerized and phosphorylated at tyrosine residues (yellow). The activated FGFRs lead to five key downstream signalling cascades: RAS-RAF-MAPK, PI3K-AKT, STAT, PLC $\gamma$ , and Src (light blue). The signalling can be negatively regulated by modulating ligand binding (e.g. sef, orange) or by modulating intracellular MAPK signalling (e.g. Sprouty, orange).

(Adapted and modified from Turner and Grose 2010)

FGFRs have several tyrosine residues in their intracellular kinase domain. Ligand binding initiates FGFR dimerization and conformational shift in receptor structure that activates the intracellular kinase domain, further leading to cross-phosphorylation of tyrosine residues within cytoplasmic tail (Turner and Grose, 2010). Some of these phosphorylated residues act as docking sites for adaptor proteins containing Src homology 2 (SH2) domains, triggering multiple downstream signalling pathways including RAS-MAPK, PI3K-AKT, signal transducer and activator of transcription (STAT), phospholipase C $\gamma$  (PLC $\gamma$ ) and Src (Turner and Grose, 2010; Figure 12). The main adaptor proteins, FGFR substrate 2 $\alpha$  and  $\beta$  (FRS2 $\alpha/\beta$ ) bind to the juxtamembrane region of FGFRs. The bound FRS2 $\alpha/\beta$  are phosphorylated by FGFRs and then associated with growth factor receptor-bound 2 (Grb2), leading to RAS-MAPK signalling that regulates cell

proliferation and differentiation, or AKT-dependent anti-apoptotic pathway. Recently it has been reported that a dimeric Grb2 can also directly bind to the two FGFR2 molecules and form a tetramer, which regulates FGFR2 activation in the presence and absence of extracellular stimuli (Lin et al., 2012). PLC $\gamma$  and Src bind to phosphotyrosine residues in the cytoplasmic tail of FGFRs. They trigger MAPK kinase signalling through PKC activation or Src signalling cascades coupled with Rho family GTPases that control cytoskeletal organization and migration (Eswarakumar et al., 2005; Turner and Grose, 2010). The FGFR downstream signalling activation is tightly controlled by a MAPK phosphatase-mediated negative feedback loop (Turner and Grose, 2010). Differences in downstream effector activation and biological functions are not dependent on specific FGF binding. This rather relies on cell type-specific adaptor protein expression and crosstalk with other signalling networks, such as Wnt signalling and HGF, PDGF or VEGF signalling (Dailey et al., 2005;

Turner and Grose, 2010). Moreover, each FGFR has a different effect on downstream signalling activation. For example, the activation of downstream targets by FGFR4 is less strong than FGFR1 (Vainikka et al., 1994) and FGFR1 signalling lasts longer than FGFR2, since FGFR2 is degraded faster than FGFR1 after activation (Xian et al., 2007).

#### **4.1.2 FGF signalling in cancer**

FGF signalling controls many fundamental cellular events in developmental processes including mesodermal patterning in embryo and subsequent formation of organs, such as skeletal development (e.g. limb and skull), the mammary and prostate gland formation as well as the nervous system generation (Eswarakumar et al., 2005; Ornitz and Marie, 2002; Turner and Grose, 2010). In adult, FGF signalling contributes to tissue homeostasis, wound healing, angiogenesis, and inflammation (Eswarakumar et al., 2005; Turner and Grose, 2010). Thus, deregulation of this signalling activation can lead to developmental disorders and cancer, as explained below (Beenken and Mohammadi, 2009; Turner and Grose, 2010; Wesche et al., 2011).

##### **4.1.2.1 Gain-of-function mutations**

Several FGFR mutations that confer constitutive kinase activation have been found in a number of congenital skeletal dysplasia. The most common genetic form of dwarfism in human, Achondroplasia, is caused by a point mutation in the transmembrane domain of FGFR3 (FGFR3-G380R). The conformational changes in the FGFR3-R380 mutant induce aberrant kinase activation through constitutive dimer formation and receptor stabilization (Cho et al., 2004; Eswarakumar et al., 2005). Moreover, point mutations in the similar transmembrane location and in extracellular Ig-III domain of FGFR1 and FGFR2, as well as another mutation in transmembrane domain, FGFR3-A391E, cause craniosynostosis that are characterized with premature fusion of skull sutures and cranial deformities (Eswarakumar et al., 2005; Meyers et al., 1995; Pulleyn et al., 1996; White et al., 2005).

These gain-of-function mutations of FGFRs have also been found in many types of cancer (Wesche et al., 2011). Glioblastoma exhibit multiple mutations in FGFR1 kinase domain (Rand et al., 2005), while FGFR2 mutants have been found in 12 % of endometrial cancer (Dutt et al., 2008) and more rarely in gastric cancer (Jang et al., 2001). In addition, various FGFR3 mutations are widely detected in many types of cancer, including bladder (50–60 % non-muscle invasive type, 10–15 % invasive type), myeloma (5 %), and prostate cancer (3 %; Turner and Grose, 2010; Wesche et al., 2011). Unlike FGFR1-3, none of FGFR4 mutations have been reported to be implicated to developmental disorders. However, several mutants are associated with progression of certain cancer. FGFR4-N535K and V550E mutations in FGFR4 tyrosine kinase domain were found in childhood soft tissue sarcoma, rhabdomyosarcoma (Taylor JG et al., 2009). More recently, a constitutive active mutant, FGFR4-Y367C in extracellular domain was identified in MDA-MB-453 breast carcinoma cells (Roidl et al., 2010). Overexpression of this mutant allows malignant cells to escape from doxorubicin treatment as well as promotes aberrant cell proliferation and tumour growth through MAPK/ERK signalling activation (Roidl et al., 2009; Roidl et al., 2010).

#### **4.1.2.2 Single nucleotide polymorphism (SNP)**

Germline SNPs have been identified in FGFRs that are associated with some types of cancer predisposition. Several SNPs in FGFR2 have been found to be highly associated with breast cancer risk (Tenhagen et al., 2012). The risk variants of FGFR2 increase the affinity to runt-related transcription factor (RUNX), resulting in increased expression of FGFR2 that associates with breast cancer development (Meyer et al., 2008; Tenhagen et al., 2012).

One SNP in the transmembrane domain of FGFR4 that leads to change of glycine to an arginine at amino acid position 388 (FGFR4-G388R) has been linked to poor prognosis of patients with several types of tumours such as breast, prostate, colon, lung, head and neck squamous cell carcinoma, high-grade soft tissue sarcoma, as well as melanomas (Bange et al., 2002; da Costa Andrade et al., 2007; Sasaki et al., 2008; Spinola et al., 2005; Streit et al., 2004; Wang et al., 2004). In contrast, in some other carcinomas including malignant gliomas and advanced ovarian cancer, this SNP is not associated with cancer progression (Marme et al., 2012; Mawrin et al., 2006). In some cases, the expression of FGFR4-R388 variant in these carcinomas is even related to prolonged survival and a better prognosis (Marme et al., 2012; Mawrin et al., 2006). The study using WAP-TGF $\alpha$  transgenic mouse carrying FGFR4-G385R mutation (analogous to the human G388R) shows it to accelerate mammary carcinoma growth and lung metastasis (Seitzer et al., 2010). This was associated with increased transformation and migration/invasion of FGFR4-G385R mouse embryonic fibroblasts (Seitzer et al., 2010). The FGFR4-R388 risk variant also promotes human prostate carcinoma cell migration through stabilization and elongation of FGF signalling compared to the alternative FGFR4-G388 low risk variant (Wang et al., 2008). In contrast to the high risk variant, the FGFR4-G388 variant expression is associated with a better prognosis of prostate and breast carcinomas (Bange et al., 2002; Stadler et al., 2006). The risk variant in breast carcinomas can be a possible marker for adjuvant systemic chemotherapy resistance (Thussbas et al., 2006). On the other hand, the FGFR4-R388 variant can be associated with better clinical and pathological response under neoadjuvant chemotherapy treatment (Marme et al., 2010).

#### **4.1.2.3 Chromosomal translocations**

*FGFR1* and *FGFR3* chromosomal translocations have been identified in hematologic malignancies, resulting in a fusion protein comprising N-terminus of a transcriptional factor fused to the C-terminus of FGFR kinase domain. The fusion protein is constitutively dimerized in the absence of ligand, resulting in activation of FGFR kinase activity (Turner and Grose, 2010). Most of FGFR1 fusion proteins were identified in patients with the myeloproliferative disorder stem cell leukaemia/lymphoma syndrome, while multiple myelomas bear *FGFR3* translocation that is associated with worse prognosis of patients (Avet-Loiseau et al., 1998; Kalff and Spencer, 2012; Turner and Grose, 2010). This translocation leads to FGFR3 overexpression through a strong IgH promoter activity (Kalff and Spencer, 2012).

#### **4.1.2.4 Gene amplification and overexpression**

*FGFR* gene amplification often leads to FGFR overexpression, which can promote ligand-independent signalling (Wesche et al., 2011). Amplification of *FGFR1* occurs in approximately 10% of estrogen receptor (ER)-positive breast carcinomas, which is linked to aggressive cancer progression and shorter overall survival (Tenhagen et al., 2012). *FGFR2* amplifications are also found in up to 10% of gastric cancers (Kunii et al., 2008). Expression levels of FGFR1 and FGFR2 are frequently increased in advanced poorly differentiated prostate carcinomas, even though amplifications of these receptors are relatively low (Kwabi-Addo et al., 2004). The mechanism of this upregulation has remained unclear so far. Amplifications of *FGFR3* have been observed rarely in cancers (Nord et al., 2010). About 10% of breast cancers display *FGFR4* amplifications, which is associated with ER and progesterone receptor (PR)-positivity and lymph node metastases (Jaakkola et al., 1993). Importantly, increased expression of *FGFR4* mRNA in ER-positive breast carcinomas is associated with poor clinical benefit with tamoxifen treatment and shorter life time after treatment (Meijer et al., 2008).

#### **4.1.2.5 Aberrant FGF signalling**

Impaired of FGFR degradation is implicated in cancer progression. Upon ligand binding, activated FGFRs are intracellularly compartmentalized and degraded in lysosomes or proteasomes, resulting in signal termination in the physiologic context (Lemmon and Schlessinger, 2010; Parachoniak and Park, 2012). Several FGFR mutants disrupt the receptor endocytic trafficking and degradation, leading to prolonged active FGF signalling (Parachoniak and Park, 2012). For example, a mutation in the transmembrane domain of FGFR3-G380R displays prolonged active FGF signalling by high recycling rate to the plasma membrane surface rather than degradation (Cho et al., 2004).

Negative regulators of FGF signalling, the Sprouty and Sef proteins are frequently down-regulated in prostate cancer, increasing FGF signalling (Darby et al., 2006; Darby et al., 2009; Fritzsche et al., 2006). Moreover, increased expression of FGF1, FGF2 and FGF7 has been detected in breast cancer stroma, which may promote tumour growth and cancer cell migration in a paracrine manner (Finak et al., 2008). Upregulation of both FGF2 and FGFR1 expression in melanoma or FGF1 and FGFR1IIIc in ovarian cancer are associated with poor patient survival by aberrant autocrine FGF signalling activation (Marek et al., 2009; Wang and Becker, 1997).

## **4.2 Eph receptor family**

The erythropoietin-producing hepatocellular (Eph) receptor family represents the largest family of receptor tyrosine kinases. Eph receptors have been classified into either EphA or EphB subfamilies based on their specific ligands. EphA receptors (EphA1-10) bind to glycosylphosphatidylinositol (GPI)-linked ephrinA ligands (ephrinA1-6), and EphB receptors (EphB1-6) to ephrinB ligands (ephrinB1-3). Exceptionally, EphA4 and EphB2 can bind to ephrinBs and ephrinA5, respectively, and EphB4 preferentially binds to ephrinB2 only (Pasquale, 2010).

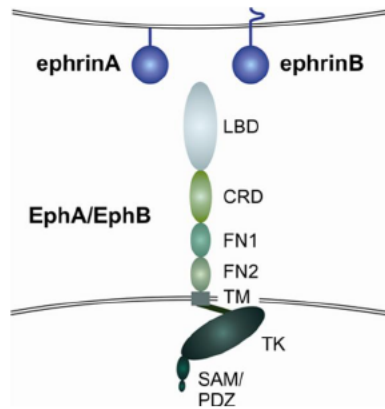


Figure 13. Structure of Eph receptors and ligands.

LBD, ligand binding domain; CRD, cysteine rich domain; FN, fibronectin type-III repeats; TM, transmembrane domain; TK, tyrosine kinase domain; SAM, a sterile alpha motif; PDZ, a post synaptic, disc large and zona occludens protein domain-binding motif.

(Adapted and modified from Murai and Pasquale, 2003)

EphA and EphB receptors share a conserved structure; the extracellular region contains a ligand-binding domain (LBD), a cysteine-rich domain (DRD) with an epidermal growth factor-like motif, and two fibronectin type-III repeats (FN1 and FN2; Figure 13). There is a single transmembrane domain and the intracellular region contains a juxtamembrane region, a TK domain, a sterile alpha motif (SAM), and a post synaptic, disc large and zona occludens protein domain-binding motif (PDZ) (Pasquale, 2010; Figure 13). In the cytoplasmic domain, the location of tyrosine residues is widely conserved within the juxtamembrane and TK domain of all Eph receptors (Binns et al., 2000). All ephrins contain a conserved extracellular N-terminal receptor binding domain. EphrinAs bind to the plasma membrane via a GPI-linkage, while ephrinBs contain a transmembrane and a short cytoplasmic sequence including PDZ domain (Pasquale, 2010).

Ephs and ephrins are expressed in almost all embryonic tissues (Miao and Wang, 2009). Since both the receptors and ligands are located on the cell surface, the Eph signalling can directly control cell-cell and cell-matrix interactions. During developmental processes, Eph signalling regulates organogenesis of vascular and nerve systems as well as the kidney, intestine, and mammary glands (Miao and Wang, 2009; Pitulescu and Adams, 2010). In adults, this signalling maintains tissue homeostasis (Miao and Wang, 2009; Pitulescu and Adams, 2010).

#### 4.2.1 Eph signalling

Upon cell-cell contact or at cell-cell junctions, the ephrin ligands bind to Eph receptors on the opposing cells, leading to the formation of a tetrameric protein complex (Himanen et al., 2007; Pasquale, 2005). In this complex, two receptors and two ligands interact via two distinct interfaces, leading to bi-directional signal transduction into both the receptor expressing cell (forward signalling) and the ligand expressing cell (reverse signalling) (Himanen et al., 2007; Pasquale, 2005). This bioactive tetramer is sequentially expanding into high-order homo or hetero Eph receptor clusters independent on additional ligand binding, which could enhance their forward signalling (Janes et al., 2012; Wimmer-Kleikamp et al., 2004). These activated receptor clustering events could regulate spatial signal restriction between cells (Nievergall et al., 2011). When both ligand and its receptor are expressed on the same cell membranes, ephrinA and EphA molecules are segregated into distinct membrane locations, which would limit the potential cis interaction on the same membrane and display either cell adhesion or cell repulsion function (Marquardt et al., 2005).

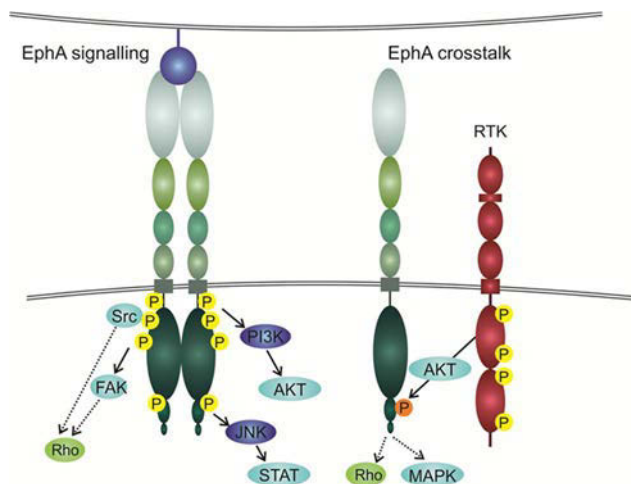


Figure 14. EphA signalling network.

(EphA signalling) Ligand binding at cell-cell contacts triggers ligand and receptor clustering and tyrosine phosphorylation (yellow) in juxtamembrane and TK domains, leading to “forward” downstream signalling cascades, e.g. PI3K-AKT, JNK-STAT, FAK, and Src (light blue). The “reverse” signalling through the ephrins can also be generated. (EphA crosstalk) The activated Akt, by other RTK signalling phosphorylates EphA2 on serine residue (orange), leading to Ras/ERK signalling and Rho-GTPase activation. (Adapted and modified from Pasquale, 2010)

Upon ligand binding at cell-cell contact, the activated Ephs trigger multiple downstream signalling pathways including PI3K-AKT, Janus kinase (JNK)-STAT, as well as FAK- and Src-Rho GTPases (Pasquale, 2008; Pasquale, 2010; Figure 14). These downstream signalling regulate cytoskeletal reorganization and cell-cell or cell-matrix interactions depending on cell type-specific adaptor protein expression and crosstalk with other signalling networks, such as other RTK signalling (e.g. EGFR, FGFR, and VEGFR) and intracellular Akt and Ras/ERK signalling pathways (Miao and Wang, 2012; Pasquale, 2010; Figure 14). In contrast, the reverse signalling in ephrin expressing cells induces SFK-dependent signalling that mediates either tight junction assembly or Rac1-mediated cytoskeletal rearrangement in a tissue context-dependent manner (Pasquale, 2010). The Eph/ephrin complexes are subsequently

removed from cell-cell contact sites by endocytosis into ephrin expressing or Eph expressing cells (Janes et al., 2012; Pasquale, 2005). For example, ADAM10 cleaves receptor-bound ephrin ligand and releases Eph/ephrin complexes from the ligand-expressing cells, which is followed by endocytosis and ultimately cell-cell repulsion (Hattori et al., 2000; Janes et al., 2005; Janes et al., 2009). The ADAM10 substrate sequence is highly conserved in the extracellular domain of all ephrins. Therefore the proteolytic regulation of ephrin/Eph complexes is a general phenomenon that regulates cell behaviour (Janes et al., 2012).

## 4.2.2 EphA2 in cancer

EphA2 is one of the best-studied Eph receptors in physiological and pathological conditions during the last decade. EphA2 is known as a tumour-suppressor, which cooperates with E-cadherin to maintain epithelial cell-cell junctions and apicobasal cellular polarity (Miura et al., 2009; Zantek et al., 1999). Aberrant expression and signalling of EphA2 have been implicated in cancer progression and poor prognosis of cancer patients (Pasquale, 2010).

### 4.2.2.1 Overexpression

EphA2 is frequently overexpressed in many types of cancer including breast, prostate, ovarian, pancreatic, colon and lung carcinomas as well as melanoma and glioblastoma multiforme (Brantley-Sieders, 2012; Margaryan et al., 2009; Wykosky and Debinski, 2008). Overexpressed EphA2 is often associated with aggressive cancer progression and poor prognosis (Brantley-Sieders, 2012; Wykosky and Debinski, 2008). In breast cancer and

glioblastoma, overexpression of EphA2 is often correlated with low expression level of its cognate ligand, ephrinA1 (Macrae et al., 2005; Wykosky et al., 2005). This imbalanced expression pattern of ligand and receptor in breast carcinoma cells is induced by the activation of Ras/ERK signalling, which is induced by other RTK signalling pathways (Macrae et al., 2005). Overexpressed EphA2 molecules on the cell surface can spontaneously associate with each other. So far this self-assembly mechanism is not fully confirmed (Himanen et al., 2007; Nievergall et al., 2011).

In normal epithelial cell layers, EphA2 signalling cooperates with E-cadherin-mediated epithelial cell-cell adhesion (Miura et al., 2009; Zantek et al., 1999). E-cadherin expression induces EphA2 localization at cell-cell junctions and increases ligand-dependent EphA2 signalling. The activated EphA2 in turn enhances E-cadherin-based cell-cell adhesion, apicobasal cellular polarity, and inhibition of actin cytoskeleton remodelling (Miura et al., 2009; Zantek et al., 1999). Overexpressed EphA2 in cancer cells disturbs epithelial adherens junctions through upregulation of Src-RhoA signalling (Fang et al., 2008; Miao et al., 2000; Parri et al., 2007; Zelinski et al., 2001). These signalling lead to suppression of integrin-mediated cell-matrix adhesion, which triggers cell-cell and cell-matrix junction disassembly and cell rounding (Fang et al., 2008; Miao et al., 2000; Parri et al., 2007; Zelinski et al., 2001). Increased RhoA activity also induces actomyosin contractility-driven amoeboid-type cell invasion (Parri et al., 2009; Taddei et al., 2011).

#### ***4.2.2.2 Ligand-independent signalling***

In advanced glioblastoma, breast and prostate carcinoma cells, the overexpressed EphA2 can cooperate with other RTK signalling in ligand-independent manner and act as a potential guidance molecule for collectively migrating cells (Miao and Wang, 2012). In the presence of the ligand, the activated EphA2 inhibits integrin-Rac-mediated cell migration/invasion and cell proliferation, accompanied by suppression of Akt-mTORC1 activities (Miao et al., 2000; Miao et al., 2009; Yang et al., 2011). In contrast, other RTK signalling, e.g. EGF signalling activates Akt that phosphorylates ligand-unbound EphA2 on a serine residue (Ser at 897) that promotes glioblastoma and metastatic prostate carcinoma cell migration/invasion (Miao et al., 2009). This serine phosphorylated EphA2 can be used as a biomarker for stratification of patients carrying brain and prostate tumours, since oncogenic Akt activation coupled with PTEN-loss is correlated with higher grade of these tumours (Miao and Wang, 2012; Parsons et al., 2008; Tomlins et al., 2007). This cross-talk is also observed in invasive breast carcinoma cells wherein EGF signalling enhances cell migration and EphA2 expression through Ras/ERK signalling, whereas ligand binding down-regulates EphA2 levels by inducing receptor internalization and degradation (Hiramoto-Yamaki et al., 2010; Macrae et al., 2005).

EphA2 overexpression can play a role in both intrinsic and acquired resistance to anti-HER2 antibody trastuzumab-based therapy that is used as an initial treatment for HER2 positive breast cancer patients (Zhuang et al., 2010). The cross-talk signalling with other RTK may be a one mechanism of cancer therapy resistance.



### **4.2.2.3 SNPs and Mutations**

Human genome sequencing studies have revealed numerous EphA2 SNP variants and somatic mutations (Greenman et al., 2007; Jun et al., 2009; Park et al., 2012; Shiels et al., 2008; Zhang et al., 2009). Several of SNPs are related to human cataract (Jun et al., 2009; Park et al., 2012; Shiels et al., 2008; Zhang et al., 2009). One of the somatic mutations identified from human cancer genomes has Gly to Arg replacement in the first FN domain (EphA2-G391R) (Faoro et al., 2010; Greenman et al., 2007). The EphA2-G391R was found in lung squamous cell carcinoma patient samples. This mutant increases cell growth and migration by focal adhesion assembly through increased EphA2 kinase activity coupled with Src, cortactin, and p130Cas downstream signalling activation (Faoro et al., 2010).

## **4.3 FGF and Eph signalling in cancer cell invasion**

Both cancer-associated FGF and Eph signalling contribute to cancer progression. Besides controlling cell proliferation and survival, these signalling promote cancer cell invasion and metastasis through regulating cytoskeletal dynamics and modulating cell-cell and cell-ECM communications (Friedl and Alexander, 2011; Lemmon and Schlessinger, 2010).

### **4.3.1 FGF signalling in cancer cell invasion**

FGF signalling can promote cell migration/invasion in several ways (Turner and Grose, 2010). For example, synthetic ligand-inducible FGFR1 (iFGFR1) signalling triggers transformations of epithelial-type prostate and breast cancer cells into motile mesenchymal phenotypes *in vitro* and *in vivo* (Acevedo et al., 2007; Welm et al., 2002; Xian et al., 2007). iFGFR1 signalling in transgenic mice further induces apoptosis-resistance by activating MAPK and Akt signalling pathways, resulting in formation of multicellular lesions in prostate and mammary epithelium that ultimately become invasive (Acevedo et al., 2007; Welm et al., 2002). In mesenchymal-type cells, FGFR forms a complex with a cell-cell and cell-matrix adhesion molecule, NCAM at the cell surface (Cavallaro et al., 2001). Through the complexes, NCAM stabilizes activated FGFR at invasive edges of tumours, leading to focal adhesion- and Rac1-mediated cancer cell invasion (Francavilla et al., 2007; Francavilla et al., 2009; Lehembre et al., 2008; Zecchini et al., 2011). Furthermore, an activation of FGF10-FGFR2 IIIb signalling in pancreatic carcinomas up-regulates MT1-MMP expression and promotes cancer invasion (Nomura et al., 2008).

FGFR4-G388R SNP is associated with aggressive cancer cell migration/invasion (Bange et al., 2002; Seitzer et al., 2010; Stadler et al., 2006; Wang et al., 2004). The homo- or heterozygous FGFR4-R388 variants are present in approximately 50% of Caucasian and Asian human population, while this variant bearing African American population is relatively low (approximately 20-30% of population; Bange et al., 2002; Wang et al., 2004; Xu et al., 2011). The FGFR4-R388 variant significantly increases prostate cancer risk in Caucasian and Asian (Ma et al., 2008; Wang et al., 2004; Xu et al., 2011), while this risk variant is rather correlated with aggressive cancer progression and poor prognosis of patients with many types of cancer including breast, prostate, and lung carcinomas (Bange et al., 2002; Spinola et al., 2005; Wang et al., 2004). To date, several cellular and molecular mechanisms of FGFR4-R388 variant-associated breast and prostate carcinoma cell migration/invasion have been

reported. This risk variant increases kinase activity in mammary tumours in transgenic mice carrying FGFR4-R385 and in prostate carcinoma cells, suggesting that the cancer-promoting potential of the FGFR4-R388 risk variant is possibly due to an enhanced kinase activity (Seitzer et al., 2010; Wang et al., 2008). This variant expression also induces upregulation of pro-migratory and pro-invasive genes including MMPs and downregulation of tumour suppressor genes (Seitzer et al., 2010; Stadler et al., 2006; Yu et al., 2011). In breast carcinoma cells, plasminogen activator inhibitor (PAI-1) expression is suppressed by the FGFR4-R388 risk variant expression, resulting in upregulation of urokinase plasminogen activator (uPA). Upregulation of uPA is linked to increase cell migration and invasion as well as decrease the sensitivity of the FGFR4-R388-expressing cells to chemotherapy-induced apoptosis (Whitley et al., 2004). In prostate carcinoma cells, upon ligand binding the stability of the activated FGFR4-R388 variant is increased and thus FGF signalling is prolonged (Wang et al., 2008). Enhanced FGFR kinase activity increases ERK/MAP signalling and transcription of pro-invasive genes, promoting prostate carcinoma cell invasion and metastasis (Wang et al., 2008; Yu et al., 2011). Thus, the kinase activities and downstream targets of the FGFR4-R388 variant seem to increase cancer cell invasion and decrease sensitivity to adjuvant chemotherapy, explaining the worse clinical outcome of carriers with this risk variant (Tenhagen et al., 2012).

#### **4.3.2 Eph signalling in cancer cell invasion**

Cancer cell dissemination from a primary tumour mass, as well as unimpeded migration and invasion through stromal space are important steps for metastatic cancer progression. Eph receptors and their membrane-anchored ligands, ephrins are located at cell-cell contact sites and directly regulate both cancer cell- cancer cell (homotypic) and cancer cell-stromal cell (heterotypic) interactions (Astin et al., 2010). Recently Astin and his colleagues reported that PC3 prostate cancer cells *in vitro* interchange between homotypic repulsion and heterotypic attraction by using different sets of Eph receptors and ligands on cancer cells and stromal cells (Astin et al., 2010). PC3 cells express both endogenous EphA and EphB receptors (EphA2, EphA4, EphB3, and EphB4) and ephrinA ligands, while fibroblasts express ephrinA and ephrinB ligands. Upon homotypic PC3 cell collision, EphA receptors are trans-activated by endogenous ephrinA ligands on the opposing cells (Astin et al., 2010). The activated EphA2 and EphA4 trigger repulsive cell movement by causing retraction of membrane protrusions and reinitiation of migration in a different direction through RhoA activation at the colliding sites (Astin et al., 2010; Figure 15A). However, heterotypic contacts between PC3 and fibroblasts triggers EphB-ephrinB signalling that can override EphA-ephrinA signalling and induces filopodia and lamellipodia formation through Cdc42 activation, resulting in unimpeded migration to the stromal space (Astin et al., 2010; Figure 15B). Thus EphB-ephrinB system can contribute to guidance of prostate cancer cell invasion into surrounding tissues, while EphA signalling contributes to cancer cell dissemination (Astin et al., 2010).

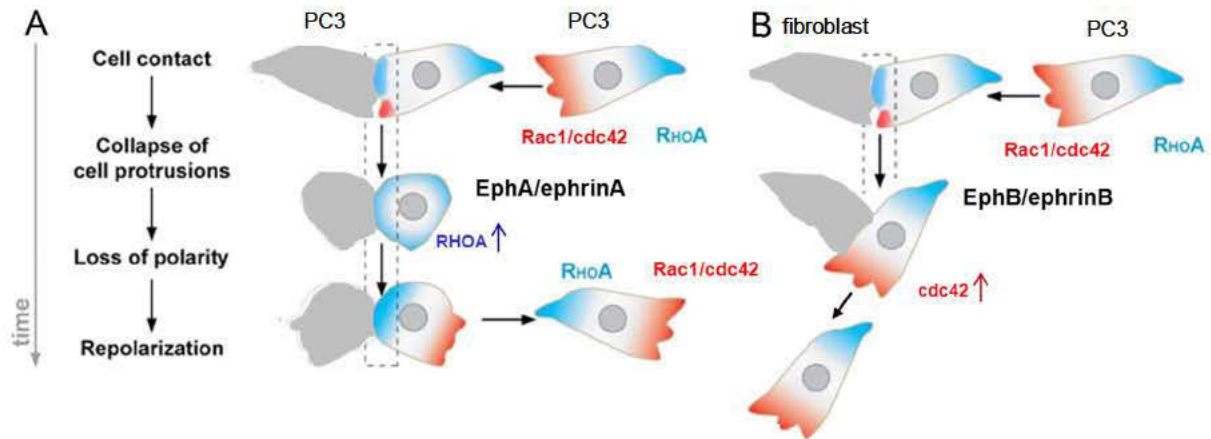


Figure 15. Eph-ephrin signalling regulates cell movement upon cell collision. (A) Upon homotypic cell collision, EphA-ephrinA signalling induces repulsive movement and dissemination of PC3 cells through RhoA activation. (B) Upon PC3-fibroblast heterotypic cell collision, EphB-ephrinB signalling promotes unimpeded migration through Cdc42 activation. (Adapted and modified from Astin et al., 2010 and Theveneau and Mayor, 2013).

Ligand-dependent EphA2 signalling triggers rapid cell rounding and homotypic cell-cell detachment in PC3 and glioma cells through Src/FAK-mediated RhoA activation concomitant with inhibition of Rac1 activity and integrin-mediated cell-matrix adhesion (Astin et al., 2010; Miao et al., 2000; Parri et al., 2007). Aberrant EphA2 overexpression in prostate carcinoma and melanoma cells triggers a transition from a mesenchymal phenotype to an amoeboid-type phenotype by RhoA activation, where EphA2 is constitutively highly phosphorylated at tyrosine residues in intracellular kinase domain (Parri et al., 2009; Taddei et al., 2011). In advanced breast carcinomas and glioblastomas, EphA2 overexpression is frequently coupled with loss or low level of ligand expression (Brantley-Sieders, 2012; Macrae et al., 2005; Wykosky et al., 2005). Ligand-independent EphA2 signalling is induced by Akt-dependent serine residue phosphorylation, instead of tyrosine phosphorylation by ephrinAs (Miao et al., 2009). This signalling leads to formation of membrane protrusion and integrin-mediated cell migration/invasion. Moreover, EphA2 ligand-independent signalling also promotes Rac1-mediated breast carcinoma cell migration toward EGF chemoattractant via activation of RhoG guanine nucleotide exchange factor, Ephexin4 (Hiramoto-Yamaki et al., 2010). Therefore a combination of expression pattern of EphA2 receptor as well as its ligands and cofactors within tumour microenvironment may control cancer cell invasion modes, e.g. amoeboid-type migration driven by RhoA-dependent actomyosin contractility or mesenchymal-type migration driven by Rac1/Cdc42-mediated cytoskeletal rearrangement coupled with integrin-mediated cell-matrix adhesion.

Overall, both FGF and Eph signalling contribute to aggressive cancer progression; however, each signalling has different functions and molecular mechanisms of cell-cell and cell-matrix communications in cancer. Understanding the different mechanisms of cancer cell invasion would help to develop new strategies for treatment of cancers driven by aberrant RTK activation.

## 5 Matrix Metalloproteinases

Cancer cell invasion drive aggressive cancer progression and poor prognosis of patients with many types of cancer. As described above, cancer-associated FGF and Eph signalling are involved in these processes. Important players for tumour expansion and metastatic cancer progression are MT-MMPs that regulate ECM remodelling by cleaving components of interstitial tissues and BMs. They also regulate cellular behaviour by modulating numerous intracellular signalling pathways through interacting with cell surface signalling receptors and intracellular cytoskeleton components.

MMPs are zinc-dependent endopeptidases composed of 23 members in vertebrates that consist of 16 soluble MMPs (MMP1-3, 7-13, 19-22, 27, and 28) and 7 membrane-anchored MMPs (MMP14-17, and 23-25; also termed MT1-MT6-MMP and MMP23) (Kessenbrock et al., 2010; Figure 16). They share a common structure, consisting of a pro-peptide, a catalytic domain, and a hemopexin-like domain that is linked to the catalytic domain via a flexible hinge region (Kessenbrock et al., 2010; Figure 16). Type I transmembrane MT-MMPs, MT1-MT3-MMP (MMP14-16), and MT5-MMP (MMP24), have a single transmembrane domain followed by a short cytoplasmic tail, whereas MT4-MMP (MMP17) and MT6-MMP (MMP25) are anchored into the plasma membrane by GPI-linker and MMP-23 is a type-II transmembrane MT-MMP that has unique cysteine array (CA) and immunoglobulin (Ig)-like domains (Kessenbrock et al., 2010; Figure 16).

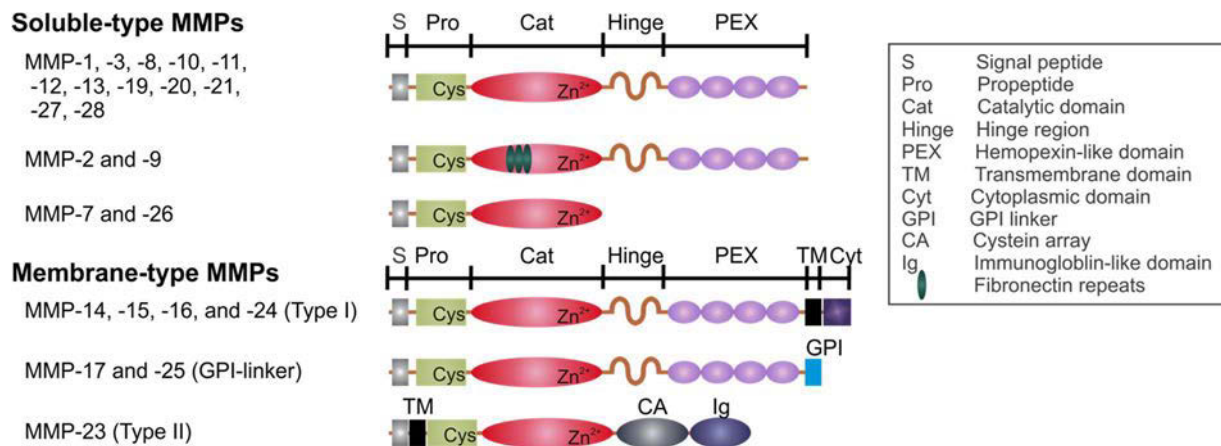


Figure 16. Structure of MMPs. (Adapted and modified from Egeblad and Werb, 2002)

MMPs are expressed in pro-forms that are kept enzymatically inactive by interaction between a cysteine residue of the pro-peptide domain and the zinc ion of the catalytic domain. The pro-peptide domain is removed by a proteolytic process, which generates catalytically active enzymes. The cleavage occurs intracellularly by a serine protease, furin, or extracellularly by other MMPs or other serine proteinases, such as plasmin (Kessenbrock et al., 2010).

### 5.1 MT-MMPs

MT1-MMP is a potent ECM remodelling enzyme that was the first reported type I transmembrane MT-MMP (Sato et al., 1994). To date, other five MT-MMPs, MT2-MMP (Will and Hinzmann, 1995), MT3-MMP (Takino et al., 1995), MT4-MMP (Puente et al.,

1996), MT5-MMP (Pei, 1999a), and MT6-MMP (Pei, 1999b) have been cloned and characterized.

In sequestered pericellular microenvironment, membrane-anchored MT-MMPs show multiple functions at the cell periphery by cleaving wide-range of ECM components and cell surface proteins (Kessenbrock et al., 2010; Page-McCaw et al., 2007; Table 1). By cleaving ECM components, MT-MMPs regulate pericellular ECM remodelling, generate functional fragments with biological activity, and release ECM-bound growth factors and cytokines. MT-MMPs also modify tissue architecture and apicobasal cellular polarity. Through interaction with cell surface receptors and intracellular cytoskeletal components, MT-MMPs further modulate intracellular signalling and cellular functions, such as cell motility, during developmental processes and in pathological conditions (Kessenbrock et al., 2010; Page-McCaw et al., 2007). Soluble-type MMPs have also similar redundant proteolytic activities in cellular microenvironment (Page-McCaw et al., 2007; Table 1).

Table 1. Substrates of MMPs. MMPs are named together with their common names.

| Name:  |                 | Substrates:   |
|--------|-----------------|---|
| MMP-1  | Collagenase-1   | Col I-III, VII, X, XI, aggrecan, fibrin, fibrinogen, fibronectin, gelatin, tenascin, laminin, nidogen, IGFBPs, vitronectin, proMMP-1 and -2, casein, pro-TNF $\alpha$ , IL-1 $\beta$ , $\alpha$ 2-M, $\alpha$ 1-PI, C1q   |
| MMP-2  | gelatinaseA     | Gelatin, elastin, fibronectin, Col I, III-V, VII, X, XI, aggrecan, fibrillin, nidogen, IGFBPs, laminin, osteonectin, osteopontin, tenascin, vitronectin, proMMP-1, -2, -8, -9, and -13, plasminogen, pro-TNF $\alpha$ , pro-IL-1 $\beta$ , $\alpha$ 2-M, $\alpha$ 1-PI, C1q                                   |
| MMP-3  | stromelysin-1   | Fibronectin, laminin, gelatin, Col I, III-V, VII, X, XI, aggrecan, elastin, decorin, nitogen, perlecan, tenascin, fibrin, fibrillin, IGFBPs vitronectin, proMMP-1, -3, -7, -8, -9, and -13, plasminogen, pro-TNF $\alpha$ , pro-IL-1 $\beta$ , VEGF-A, proHB-EGF, CTGF, E-cadherin, $\alpha$ 2-M, C1q         |
| MMP-7  | matrilysin      | Fibronectin, laminin, Col IV, gelatin, aggrecan, decorin, nidogen, elastin, fibrillin, laminin, MBP, proMMP-2 and -7, E-cadherin, pro- $\alpha$ -defensin, casein, pro-TNF $\alpha$ , plasminogen, integrin $\beta$ 4, syndecan-1, $\alpha$ 1-PI  |
| MMP-8  | collagenase-2   | Col I-III, gelatin, aggrecan, fibrinogen, proMMP-8, tenascin, $\alpha$ 2-M,   |
| MMP-9  | gelatinaseB     | Gelatin, Col IV, V, VII, XI, XIV, XVII, elastin, fibrillin, fibronectin, aggrecan, fibrin, MBP, decorin, laminin, osteonectin, vitronectin, IGFBPs, $\alpha$ 2-M, $\alpha$ 1-PI, casein, pro-TNF $\alpha$ , pro-IL-1 $\beta$ , plasminogen, E-cadherin, dystroglycan, C1q                                     |
| MMP-10 | stromelysin-2   | Fibronectin, laminin, gelatin, Col III-V, aggrecan, fibrinogen, proMMP-1, -2, -8, and -13, casein   |
| MMP-11 | stromelysin-3   | Laminin, fibronectin, aggrecan, IGFBPs, $\alpha$ 1-PI   |
| MMP-12 | metalloelastase | Col IV, elastin, fibronectin, fibrin, laminin, nidogen, fibrillin, vitronectin, proMMP-2, pro-TNF $\alpha$ , $\alpha$ 2-M, $\alpha$ 1-PI, plasminogen   |
| MMP-13 | collagenase-3   | Col I-VI, IX, X, XIV, aggrecan, fibrillin, fibronectin, gelatin, fibrinogen, proMMP-2, -9 and -13, $\alpha$ 2-M, $\alpha$ 1-PI, casein  |
| MMP-14 | MT1-MMP         | Col I-IV, gelatin, fibronectin, laminin, vitronectin, aggrecan, tenascin, nidogen, perlecan, fibrin, fibrillin, proMMP-2 and -13, MMP-16 and -17, $\alpha$ 2-M, $\alpha$ 1-PI, CD44, LTBP, pro-TNF $\alpha$ , IL-8, CXCL12, integrin- $\alpha$ v, syndecan-1, EMMPRIN, E- and N-cadherins, ICAM-1, ADAM9, LRP |
| MMP-15 | MT2-MMP         | Col I, IV, gelatin, fibronectin, laminin, vitronectin, aggrecan, tenascin, nidogen, perlecan, fibrin, proMMP-2, tTg   |
| MMP-16 | MT3-MMP         | Col III, IV, gelatin, fibronectin, laminin, vitronectin, aggrecan, fibrin, proMMP-2, MMP-14, CD44, APP, NgR1, syndecan-1, tTg, ICAM-1   |
| MMP-17 | MT4-MMP         | Gelatin, fibrillin, fibronectin, pro-TNF $\alpha$ , ADAM-TS4  |

|        |              |  |
|--------|--------------|--|
| MMP-19 |              | Col IV, gelatin, fibronectin, tenascin, aggrecan, COMP, casein   |
| MMP-20 | enamelysin   | Amelogenin, aggrecan, COMP   |
| MMP-21 |              | $\alpha$ 1-PI  |
| MMP-23 |              | gelatin  |
| MMP-24 | MT5-MMP      | Heparan and Chondroitin sulphate proteoglycans, gelatin, fibronectin, proMMP-2, APP  |
| MMP-25 | MT6-MMP      | Col IV, gelatin, fibrin, fibronectin, vitronectin, proMMP-9, MBP, $\alpha$ 1-PI  |
| MMP-26 | matrilysin-2 | Col IV, gelatin, fibronectin, fibrin, laminin, nidogen, fibrillin, vitronectin, plasminogen, casein, proTNF $\alpha$ , $\alpha$ 2-M, $\alpha$ 1-PI |
| MMP-27 |              | Casein   |
| MMP-28 | epilysin     | Casein, NCAM   |

Abbreviations:  $\alpha$ 1-PI,  $\alpha$ 1 proteinase inhibitor;  $\alpha$ 2-M,  $\alpha$ 2-macroglobulin; APP, amyloid  $\beta$ A4 protein; C1q, complement C1q; Col, collagen; COMP, cartilage oligomeric matrix protein; CTGF, connective tissue growth factor; HB-EGF, heparin-binding EGF-like growth factor; IGFBP, insulin-like growth factor binding protein; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-8, interleukin-8; MBP, myelin basic protein; LRP1, low-density lipoprotein receptor related protein; LTBP, latent transforming growth factor- $\beta$ (TGF- $\beta$ ) binding proteins; NgR, Nogo-66 receptor-1; tTg, tissue transglutaminase

### 5.1.1 MT-MMP activity

In tumour microenvironment, MMPs are expressed by cancer-associated stromal cells including CAFs, endothelial cells, neutrophils, lymphocytes, and macrophages, which can promote tumour progression (Kessenbrock et al., 2010). Invading cancer cells also use the proteolytic activities of MMPs to infiltrate into adjacent ECM by breaking of physical ECM barriers, e.g. dense sheet-like BMs and mesh-work interstitial matrix (Rowe and Weiss, 2008; Rowe and Weiss, 2009).

In a primary tumour, epithelial-type carcinoma cells loosen cell-cell adhesion and adapt a tissue-invasive mesenchymal phenotype (Yilmaz and Christofori, 2010). Mesenchymal-type cells express several MMPs including MMP1, MMP9, MT1-MMP and MT2-MMP (Miyoshi et al., 2004; Tao et al., 2011; Vandewalle et al., 2009). Expressed MMPs, such as MMP9 and MT1-MMP cleave E-cadherin and further dissociate epithelial cell junctions and disturb apicobasal cell polarity (Covington et al., 2006; Cowden Dahl et al., 2008; Figure 17). At the important first step for local invasion, cancer cells degrade BMs to infiltrate into the adjacent interstitial matrix (Kalluri, 2003; Rowe and Weiss, 2008). MT1-MMP and MT2-MMP potently break BMs by cleaving of collagen IV and laminin (Hotary et al., 2006; Ota et al., 2009; Figure 17). Thus, these enzymes expressing breast carcinoma cells can transmigrate through BMs. Although MT3-MMP displays BM degradation in ex vivo model (Hotary et al., 2006), its activity is not confirmed in other models (Ota et al., 2009). This difference may reflect the differential regulation of MT3-MMP activity in variable microenvironments. MMP2 and MMP9 can act as type-IV collagenases (Itoh and Seiki, 2006). However, recent studies have questioned the importance of both MMPs in BM transmigration (Hotary et al., 2006; Ota et al., 2010). The activities of MT4-MMP, MT5-MMP, and MT6-MMP on BM breaching are negligible (Hotary et al., 2006).

In the interstitial tissues, infiltrating mesenchymal-type cancer cells use membrane-anchored and soluble MMPs for invasion. MT1-MMP, the most widely expressed member of the MT-MMP family, is a critical enzyme for interstitial tissue remodelling by cleaving the main ECM component, type I collagen, and also type II and III collagens, gelatin, fibronectin,

vitronectin, aggrecan, and fibrin (Itoh and Seiki, 2006; Table 1 and Figure 17). MT1-MMP is expressed at the leading edge of leader cells of collective cell cohort and mesenchymal-type singly-invading cells. The proteolytic activity of this enzyme assists in forming de-novo gaps and micro-tracks for cancer invasion (Friedl and Alexander, 2011; Sabeh et al., 2009; Wolf et al., 2007). Unlike MT1-MMP, MT3-MMP does not degrade cross-linked collagen efficiently (Li and Zhu, 2010; Sabeh et al., 2004; Shimada et al., 1999). MT3-MMP rather cleaves fibrin, and importantly this enzyme reduces MT1-MMP expression on the cell surface (Hotary et al., 2002; Tatti et al., 2011; Table 1).

By cleaving ECM components, MMPs generate biologically functional ECM fragments that can promote cell-matrix adhesion or cell migration (Kessenbrock et al., 2010; Page-McCaw et al., 2007). For example,  $\gamma 2$  and  $\beta 3$  subunit fragments of laminin 5 cleaved by MT1-MMP promote cell migration and invasion *in vitro* and *in vivo* (Giannelli et al., 1997; Gilles et al., 2001; Hamasaki et al., 2011; Koshikawa et al., 2000; Udayakumar et al., 2003). MMPs also release ECM-bound growth factors and cytokines, e.g. EGF (Koshikawa et al., 2011), TNF- $\alpha$  (Tam et al., 2004), and LTBP-bound TGF- $\beta$  (Mu et al., 2002; Tatti et al., 2008; Figure 17), and thus increase the bioavailability of these soluble factors in extracellular milieu. Furthermore, MMPs can also modulate the activities of growth factors and cytokines by cleavage, e.g.  $\alpha 1$ -proteinase inhibitor inactivation ( $\alpha 1$ -PI; Kataoka et al., 1999), IL-8 and CXCs degradation (Tam et al., 2004; Dean et al., 2008; Tam et al., 2004; Table 1).

On the cell surface, MT-MMPs regulate activities of soluble-type MMPs and the related metzincin proteases, a disintegrin and metalloproteinase (ADAM) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAM-TS) (Chan et al., 2012; Kessenbrock et al., 2010; Rodriguez-Manzanera et al., 2000). For examples, MT1-MMP is a main activator for latent-type MMP2 (Lehti et al., 1998; Sato et al., 1996b). Tissue inhibitor of metalloproteinases 2 (TIMP2) can assist in this process. The catalytic domain of MT1-MMP binds to the N-terminal domain of TIMP2 and the C-terminal domain of TIMP2 binds to the hemopexin domain of proMMP2, forming an MT1-MMP-TIMP2-MMP2 complex (Strongin et al., 1995). The N-terminal pro-peptide domain of proMMP2 is cleaved by a TIMP2-free adjacent MT1-MMP, generating an active form of MMP2 (Itoh et al., 2001; Lehti et al., 2002; Sato et al., 1996b). MT1-MMP also activates proMMP13 and inhibits ADAM9 activity by shedding it (Chan et al., 2012; Figure 17).

Furthermore, MT1-MMP mediates cellular functions by modulating many intracellular signalling pathways through interacting and cleaving cell surface adhesion molecules, e.g. CD44 (Kajita et al., 2001), low-density lipoprotein receptor related protein (LRP1; Lehti et al., 2009; Rozanov et al., 2004),  $\alpha v$  integrin (Deryugina et al., 2002), and syndecan-1 (Endo et al., 2003; Table 1 and Figure 17), promoting cancer cell motility. Interestingly, MT1-MMP forms complexes with PDGFR $\beta$  and FGFR2 on the cell surface, which can indirectly modulate these RTK signalling activities by cleaving their co-factors, LRP1 and ADAM9, respectively (Chan et al., 2012; Lehti et al., 2005; Lehti et al., 2009; Figure 17). The short cytoplasmic domains of MT-MMPs can associate with cytoskeleton components, promoting cell invasion through confined MT1-MMP localization at invasive membrane protrusions (Nakahara et al., 1997; Poincloux et al., 2011). Furthermore, phosphorylation of a tyrosine residue (Tyr at 573) in MT1-MMP cytoplasmic domain by non-receptor tyrosine kinase, Src

is also essential for effective cancer cell invasion (Labrecque et al., 2004; Moss et al., 2009; Nyalendo et al., 2007; Nyalendo et al., 2008; Figure 17).

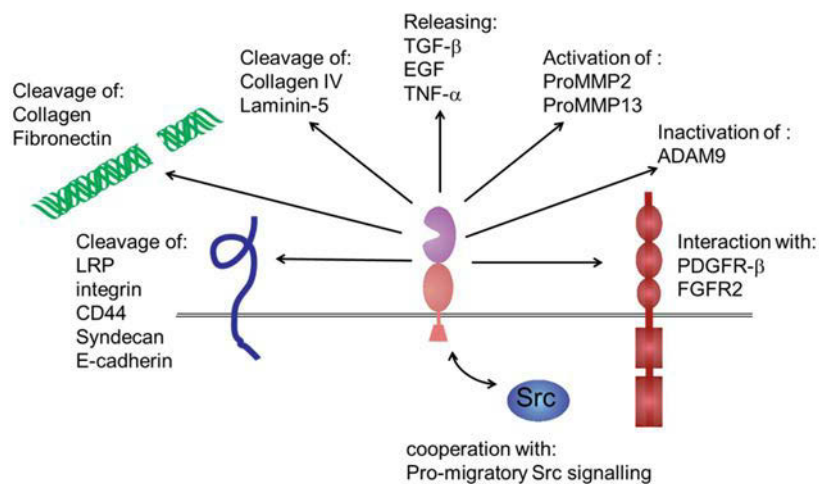


Figure 17. Biological functions of MT1-MMP. These functions promote cancer cell invasion and migration. (Adapted and modified from Ito and Seiki, 2006).

Although the substrates of MMPs overlap, the specificity of substrate for individual MMPs relies on the sequence of their catalytic domains (substrate-binding pocket) that recognize the exposed specific substrate sequence (Overall, 2002). MMPs bind to the specific substrates and inhibitors through exosites located on the hemopexin domain (Overall, 2002). The interaction of MMP and their substrates is also supported by binding through hinge region (Osenkowski et al., 2005).

### 5.1.2 Regulation of MT-MMP activity

Due to the multiple functions of MT-MMPs within pericellular microenvironment, their temporal and spatial expression, activation, and localization are tightly controlled by both transcriptional and posttranscriptional processes (Egeblad and Werb, 2002; Kessenbrock et al., 2010).

#### 5.1.2.1 Transcriptional regulation

Transcriptional regulation of *MMP* expression is mediated by several growth factor/cytokine signalling pathways. These signalling pathways associated with the induction of EMT (e.g. TGF- $\beta$ , Wnt, and TNF- $\alpha$ ) can trigger the expression of *MT1-MMP* and other ones such as *MMP3*, *MMP7*, *MMP9*, and *MT2-MMP* (Blavier et al., 2006; Labbe et al., 2007; Ottaviano et al., 2006; Takahashi et al., 2002; Udayakumar et al., 2003). E-cadherin repressor, Snail and hNanos1 can up-regulate *MT1-MMP*, and the expressed MT1-MMP cleaves E-cadherin and eventually dissociates E-cadherin-mediated adherence junctions (Bonnomet et al., 2008; Miyoshi et al., 2004). Phorbol 12-myristate 13-acetate (PMA) and concanavalin A treatment also induces *MT1-MMP* transcription (Lohi et al., 1996). Moreover, *in vivo* and within 3D collagen matrix type I collagen induces cell-surface MT1-MMP expression transcriptionally through TGF- $\beta$  signalling (Ottaviano et al., 2006; Sakai et al., 2011; Shields et al., 2011).



### 5.1.2.2 MMP inhibitors

The MMP activities on the cell surface or in the extracellular milieu are constantly controlled by the endogenous MMP inhibitors. The major inhibitor in tissue fluid is the abundant plasma protein  $\alpha$ 2-macroglobulin ( $\alpha$ 2-MG), which efficiently binds to the active site of MMPs. The complexes of  $\alpha$ 2-MG-MMP are then bound to scavenger receptors and irreversibly cleared by endocytosis (Kessenbrock et al., 2010). A reversion-inducing-cysteine rich protein with Kazal motifs (RECK) also inhibits MT-MMPs and MMP2 proteolytic activities (Oh et al., 2001).

The most potent MMP inhibitors are tissue inhibitors of metalloproteinases (TIMPs). They are secreted proteins that reversibly bind to MMPs at a 1:1 ratio and inhibit their activities (Brew and Nagase, 2010; Lambert et al., 2004). Unlike  $\alpha$ 2-MG, four members of TIMPs (TIMP1-4) are expressed in specific tissues and have different binding specificities to individual MMPs, as well as ADAMs and ADAMTSs (Lambert et al., 2004). The C-terminal domain of TIMP2 binds to the hemopexin domain of the proenzyme of MMP2, which is essential for the cell surface MMP2 activation by MT1-MMP (Strongin et al., 1995). TIMPs 2-4 bind to soluble MMPs and MT-MMPs, whereas TIMP1 has more restricted specificity and does not bind to MT-MMPs (Lambert et al., 2004). The balance of activities between TIMPs and MMPs are important for tissue homeostasis. Therefore, the disruption of this balance can be implicated in cancer progression.

### 5.1.2.3 Intracellular trafficking and cell-surface localization of MT-MMPs

The localization and compartmentalization of MMPs are important for temporal and spatial restriction of biological MMP activities. The localization of MMPs to specific sites of plasma membrane is regulated by exocytotic transportation and interaction with cytoskeleton or cell surface receptors such as integrins (Poincloux et al., 2009; Rowe and Weiss, 2009). This increases their concentration and proteolytic activities in the limited pericellular environment and may also allow escaping from accessibility of their inhibitors (Kessenbrock et al., 2010). Confined localization of MT-MMPs and several soluble type MMPs on the invasive membrane protrusions is critical for their ability to drive cell invasion (Bourguignon et al., 1998; Brooks et al., 1996; Hotary et al., 2000; Rupp et al., 2008).

In the case of MT1-MMP, the expressed ~63 kDa proMT1-MMP is converted to a catalytically active enzyme (~60 kDa) by furin cleavage in trans-Golgi network (TGN) prior to trafficking to the cell surface (Lehti et al., 1998; Mazzone et al., 2004; Sato et al., 1996a; Yana and Weiss, 2000). Following activation, MT1-MMP is translocated to intracellular Rab8-positive exocytotic vesicles that actively transport MT1-MMP to invasive membrane protrusions, such as invadopodia (Bravo-Cordero et al., 2007; Figure 18). Invadopodia is a small dot-shaped actin-based invasive structure on the basal surface of invading cells, where cell-ECM adhesion molecules (e.g. integrin), tyrosine kinases (e.g. FAK and Src), actin-assembly regulators (e.g. cortactin and Arp2/3) and proteases are concentrated (Murphy and Courtneidge, 2011; Poincloux et al., 2009; Rowe and Weiss, 2009). Rho-GTPases mediate polarized trafficking of MT1-MMP-containing vesicles to invadopodia (Itoh et al., 2008; Itoh et al., 2011; Sakurai-Yageta et al., 2008). The vesicles are then fused to invadopodial

membranes and MT1-MMP is ultimately located to the tip of the protrusion structure through the interaction with  $\beta$ 1 integrin and cortactin (Murphy and Courtneidge, 2011; Poincloux et al., 2009; Rowe and Weiss, 2009). Soluble-type MMP2 and MMP9 are also specifically located at invadopodia, where MMP2 binds to integrin  $\alpha\beta$ 3 (Brooks et al., 1996; Rupp et al., 2008) and MMP9 to CD44 (Bourguignon et al., 1998). The accumulated MT1-MMP can activate latent-type MMP2 in these structures, amplifying pericellular proteolytic activities and promoting cancer cell invasion (Lehti et al., 1998; Sato et al., 1996b; Rupp et al., 2008).

On the cell surface, MT1-MMP activity is further regulated by proteolytic shedding, autocatalytic inactivation, endocytosis, and exosome secretion (Hakulinen et al., 2008; Jiang et al., 2001; Lehti et al., 1998; Lehti et al., 2000). The ~60 kDa active MT1-MMP on the cell surface forms a homo-oligomer via hemopexin domain and/or transmembrane domain (Itoh et al., 2001; Itoh et al., 2008; Lehti et al., 2002). Within the oligomers, the active form of MT1-MMP is subsequently autocatalytically processed to ~43 kDa form that lacks the catalytic domain (Lehti et al., 1998; Lehti et al., 2000; Stanton et al., 1998; Toth et al., 2002). The appearance of this catalytically inactive form can be an indication of functional activity of MT1-MMP on the cell surface, since a high level of ~43kDa form coincides with high proMMP2 activation (Lehti et al., 1998; Stanton et al., 1998). MT1-MMP is also able to be processed within hemopexin domain and in stem region (between hemopexin and transmembrane domains), resulting in soluble functional ~50kDa, ~40kDa, and ~32kDa fragment generation in extracellular milieu (Toth et al., 2002; Toth et al., 2005).

Cell surface MT1-MMP is constantly compartmentalized into intracellular endosomes. This event is regulated by both clathrin- and caveolae-dependent pathways (Jiang et al., 2001; Labrecque et al., 2004; Remacle et al., 2003; Uekita et al., 2001; Zucker et al., 2002; Figure 18). By endocytosis, the inactivated MT1-MMP is cleaned from cell surface (Maquoui et al., 2000; Zucker et al., 2002; Figure 16). A dileucine motif, LLY sequence in the cytoplasmic tail of MT1-MMP is a functional binding site for the AP-2-clathrin adaptor complex (Uekita et al., 2001). Phosphorylation of this tyrosine (Tyr at 573) by Src is essential for clathrin-dependent MT1-MMP endocytosis (Nyalendo et al., 2007). Of note, impaired MT1-MMP phosphorylation stabilizes MT1-MMP on the cell surface, leading to induction of EMT, activation of ovarian cancer cell invasion, and reduction of cell proliferation within 3D collagen matrix (Moss et al., 2009). The cytoplasmic tail of MT1-MMP can also associate with caveolin-1 in the lipid-rafts on cancer cells and endothelial cells (Annabi et al., 2001; Galvez et al., 2004; Remacle et al., 2003). MT1-MMP is thus endocytosed by caveolae-dependent pathway (Labrecque et al., 2004; Remacle et al., 2003)

The internalized MT1-MMPs are then transported to lysosomal degradation or recycling compartments that potentially relocate to the plasma membrane (Remacle et al., 2003; Wang et al., 2004; Figure 18). MT1-MMP recycling via TGN requires the presence of DKV motif in its cytoplasmic domain, and EWV motif for other MT2, MT3, and MT5-MMPs (Wang et al., 2004). Taken together, the newly synthesized MT1-MMP and recycled pre-existing MT-MMPs are actively delivered to invadopodia and other invasive membrane protrusion, where they can play proteolytic activities and further drive cell invasion (Poincloux et al., 2009).

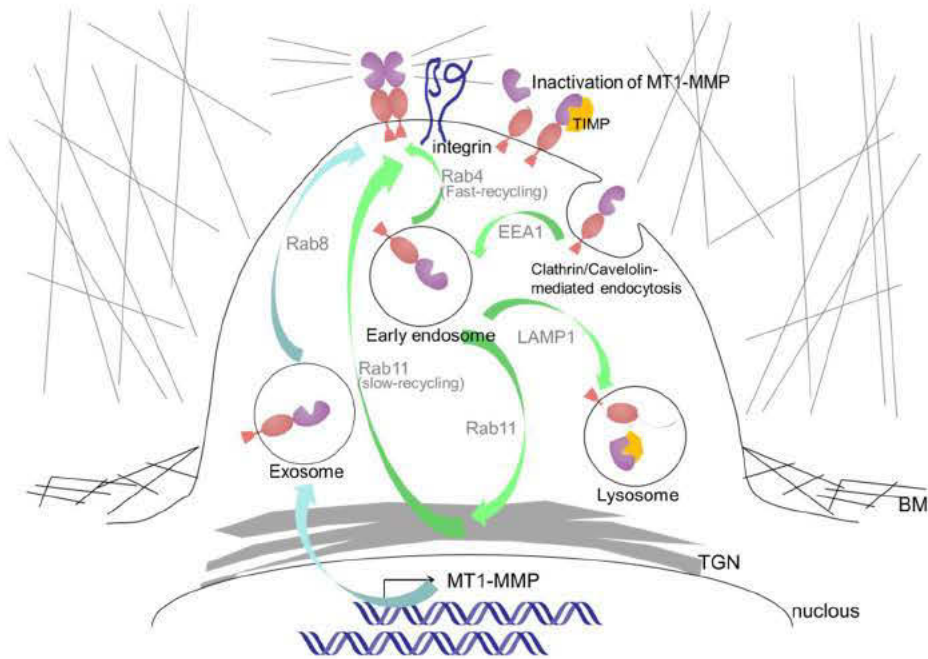


Figure 18. Regulation of MT1-MMP activity by intracellular trafficking and cell-surface localization. Blue lines indicate the traffic of the newly synthesized MT1-MMP and green lines indicate the trafficking after caveolin- or clathrin-mediated endocytosis. (Adapted and modified from Ito and Seiki, 2006; Poincloux et al., 2009; Rowe and Weiss, 2009).

## 5.2 Functions of MT1-MMP

### 5.2.1.1 MT1-MMP function in development

The functional activities of MT1-MMP are emphasized in the results from a genetic *MT1-MMP* deficient mouse model. To date, at least 15 *MMP* gene deficient mice have been generated and characterized (Page-McCaw et al., 2007). Among these single *MMP* gene knockout mice, the *MT1-MMP* knockout mice show the most severe phenotype characterized by smaller body size and lighter weight coupled with defective bone growth and neovascular formation as well as premature death after 3 to 12 weeks after birth (Holmbeck et al., 1999; Zhou et al., 2000). The observed phenotypes of MT1-MMP knockout mice reflect fundamental physiological functions of MT1-MMP on collagen remodelling (Page-McCaw et al., 2007). Moreover, MT1-MMP and MMP2 double knockout mice display lethal phenotype at birth (Oh et al., 2004). Although MMP2 single knockout mice display mild skeletal defect, they are viable and fertile (Inoue et al., 2006), MMP2-depletion therefore enhances the defects in MT1-MMP knockout mice. It suggests that MT1-MMP and MMP2 share redundant functions in ECM remodelling. Overall mouse mutation studies provide insights into MT1-MMP functions that are not required during embryonic development. MT1-MMP rather specifically acts a critical role in postnatal mammary, skeletal, and vascular system development and homeostasis (Page-McCaw et al., 2007).

Several studies have provided the molecular basis of MT1-MMP function that is co-operating with RTK signalling pathways in organ development. Young MT1-MMP deficient mice display hypoplastic architecture of the aorta (Lehti et al., 2005). The similar phenotype is observed in PDGF-B or PDGF receptor  $\beta$  knockout mice (Crosby et al., 1998; Hellstrom et

al., 2001). Consistently, PDGF signalling is attenuated in MT1-MMP knockout mice (Lehti et al., 2005). MT1-MMP associates with PDGFR $\beta$ , integrin  $\beta$ 3, and LRP1 on the same membrane surface of vascular smooth muscle cells, promoting PDGF-BB-induced signalling via the receptor by cleaving a negative regulator of PDGFR signalling, LRP1. Through this complex MT1-MMP regulates vessel wall architecture maturation and vascular smooth muscle cell dedifferentiation *in vivo* (Lehti et al., 2005; Lehti et al., 2009). Interestingly, MT1-MMP deficient mice also display similar phenotypes with FGF signalling mutants, e.g. defective calvarial bone growth in FGF18 knockout mice (Liu et al., 2002; Ohbayashi et al., 2002). Recently, it was reported that MT1-MMP forms a complex with ADAM9 and FGFR2 on the cell surface in osteoblasts and proteolytically inhibits ADAM9 activity, which sustains FGFR signalling by protecting of FGFR2 from ADAM9-mediated cleavage and rescues calvarial osteogenesis (Chan et al., 2012). Therefore, MT1-MMP indirectly modulates PDGF and FGF receptor tyrosine kinase signalling pathways by cleaving their co-factors in the same protein complexes on the cell surface *in vivo* (Chan et al., 2012; Lehti et al., 2005; Lehti et al., 2009).

### **5.2.1.2 MT1-MMP in cancer cell invasion and metastasis**

Some studies using *in vitro* cross-linked collagen matrix and *in vivo* mouse xenograft revealed that ectopic overexpression of MT1-MMP accelerates the invasive growth of poorly invasive squamous cell carcinoma cells (SCCs; Hotary et al., 2003). Conversely, fibroblasts and invasive human fibrosarcoma cells (HT-1080) that normally express high levels of MT1-MMP fail to invade into thick cross-linked collagen matrix after MT1-MMP knockdown (Sabeh et al., 2004; Sabeh et al., 2009). Inhibition of MT1-MMP by a monoclonal antibody blocks breast carcinoma cell growth, invasion, and angiogenesis in a mouse xenograft model (Devy et al., 2009). Together, these studies suggest that this protease plays multifaceted critical roles in malignant cancer progression.

Upregulation of MT1-MMP is frequently observed in many types of human cancer, where MT1-MMP expression is induced at invasive edges of tumours and reactive stroma (Okada et al., 1995; Szabova et al., 2005). In invasive carcinoma cells, MT1-MMP is associated with  $\beta$ 1 integrin in invadopodia or other invasive protrusive structures that interact with collagen fibres along the leading edge, where both cell traction and ECM degradation take place to drive mesenchymal-type cancer cell invasion (Packard et al., 2009; Wolf et al., 2007). When MT1-MMP activity is inhibited by inhibitors or gene knockdown, cancer cells can switch their invasive machinery from a protease-dependent mesenchymal phenotype to a protease-independent amoeboid-type phenotype that is driven by RhoA-mediated actomyosin contractility (Sabeh et al., 2009; Wolf et al., 2003). The population of amoeboid-type invading cells are minor, but observed in many types of tumour. They infiltrate rapidly through pre-existing matrix-free spaces that are generated by proteolytic activities of cancer-associated stromal cells or by aberrant ECM metabolism (Levental et al., 2009; Sabeh et al., 2009; Sanz-Moreno and Marshall, 2010; Wolf et al., 2003; Wolf and Friedl, 2011). For example, the activated CAFs express MT1-MMP to degrade adjacent ECM components and generate matrix-free micro-tracks which can be utilized by collective carcinoma cell invasion and protease-independent cell invasion (Chaudhry et al., 2013; Gaggioli et al., 2007).

Besides adjacent ECM degradation, MT1-MMP can promote cancer-associated new blood vessel formation in a paracrine manner by increased pro-angiogenic VEGF-A expression in carcinoma cells through Src, Akt, and mTOR signalling activation (Eisenach et al., 2010; Sounni et al., 2004). On endothelial cells, MT1-MMP sheds and decreases membrane-anchored Tie-2 receptor levels, resulting in vessel destabilization and subsequent endothelial migration (Onimaru et al., 2010), whereas endoglin cleavage inhibits angiogenesis (Hawinkels et al., 2010). Cleavage of LTBP by MT1-MMP in perivascular stroma increases TGF- $\beta$  bioavailability and its signalling via the cell-surface receptors on endothelial cells, leading to new vessel formation (Sounni et al., 2011; Tatti et al., 2008). Cancer-associated vessels are immature and highly permeable. These circulation systems can be thus used as matrix-free passage-way for metastatic cancer cells.

Taken together, the membrane-anchored MT1-MMP can function in sequestered pericellular microenvironment to drive cancer cell invasion and metastasis. By cleaving adjacent ECM components, MT1-MMP promotes cancer cell invasion through basement membrane and native cross-linked fibrillar collagen matrices. This collagenolytic activity also provides poorly cross-linked reconstituted environment that allows cells to rapidly migrate by squeezing through small spaces within matrix. Furthermore, by activation of pro-angiogenic signalling, MT1-MMP promotes angiogenesis and further facilitates cancer cell dissemination from a primary tumour to distant organs. Therefore, understanding of the molecular mechanisms of this multi-functional protease may help to develop strategies for efficient clinical interventions for cancer patients.

## **AIMS OF THE STUDY**

Cancer cell invasion includes orchestrated processes that are driven by protein kinase signalling and pericellular protease activities. Membrane-type1 matrix metalloproteinase (MT1-MMP) plays multifaceted critical roles in cancer cell invasion and metastasis. In sequestered pericellular microenvironment, this protease potently regulates pericellular ECM remodelling and modulates motile cell behaviour by cooperating with numerous protein kinase signalling pathways. The main aim of this study was to define upstream or cooperating protein kinase signalling that regulate pro-invasive MT1-MMP activities in cancer progression. I first carried out a screen to identify cellular kinases that are involved in MT1-MMP function in cancer cell invasion. Among the identified novel MT1-MMP regulators I focused on FGFR and EphA2, since the cell surface receptor kinases can be potential therapeutic targets for patients with cancer. Therefore, I continued to explore cooperative molecular mechanisms linking cancer-associated FGFR and Eph receptor tyrosine kinase signalling to the pro-invasive activities of MT1-MMP.

The specific aims of this study were:

- 1) To identify cancer-associated upstream and co-operative signalling kinases for pro-invasive MT1-MMP activities and cancer cell invasion.
- 2) To define the molecular mechanisms of co-operating fibroblast growth factor receptor-4 (FGFR4) signalling with MT1-MMP in cancer progression.
- 3) To define how the MT1-MMP-EphA2 axis regulates cell-cell communications and different invasion modes in cancer.

## MATERIALS AND METHODS

The methods used in this study are described in “Materials and Methods” section of each original publication I, II, and III. The experimental procedures that required laboratory animal usage were approved by the State Provincial Office of Southern Finland and performed according to ethical guidelines of European Convention.

### 1 Methods used in this study

| Method:   | Used in:   |
|---|------------|
| 3-dimentional type I collagen invasive growth assay   | II, III    |
| 3-dimentional type I collagen invasion assay  | I, II, III |
| cell surface biotinylation assay  | I, III     |
| FGFR4 allele genotyping from cultured cells, human skin samples, and human breast carcinoma samples | I, II      |
| fluorescent-labelled matrix degradation assay   | I          |
| gelatin zymography  | I, III     |
| gene expression database analysis (gene sapiens)  | II         |
| gene knockdown by RNA interference  | I, II, III |
| histology   | II, III    |
| immunoblotting  | I, II, III |
| immunofluorescence of cultured cells and tissues  | I, II, III |
| immunohistochemistry  | II, III    |
| immunoprecipitation   | I, III     |
| kinase screen   | I          |
| mammalian cell culture  | I, II, III |
| mass spectrometry   | III        |
| microscopy (epifluorescence and confocal microscopy)  | I, II, III |
| plasmid cDNA mutagenesis  | I, III     |
| real-time quantitative PCR (qPCR)   | I, II, III |
| Rho-GTPases activity assay  | III        |
| RNA extraction and reverse transcription  | I, II, III |
| SDS-PAGE  | I, II, III |
| statistical analysis  | I, II, III |
| time-laps imaging and cell detachment and cell-cell repulsion analyses                              | III        |
| transfection of cells   | I, II, III |
| xenograft tumour growth of human breast and prostate carcinoma cells in mouse                       | II, III    |

### 2 Cell culture and reagents (I, II, III)

All mammalian cells were grown in Dulbecco’s modified Eagle’s medium (DMEM), Minimal Eagle’s essential medium (MEM), or RPMI-1640 medium containing 10 % (v/v) heat inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, 100 µg/ml streptomycin,

and 2 mM L- glutamine (Sigma). All cells were grown to confluency at 37 °C in a humidified 5 % CO<sub>2</sub> atmosphere.

| Cell line: | Description:                                  | Medium: | Source:  | FGFR4 allele type: | Used in:   |
|------------|---|---------|----------|--------------------|------------|
| COS-1      | monkey kidney cell line                       | DMEM    | ATCC     | -                  | I, III     |
| HT-1080    | human fibrosarcoma cell line                  | MEM     | ATCC     | G/G                | I          |
| ScC-25     | human human oral squamous carcinoma cell line | DMEM    | ATCC     | G/G                | II         |
| PC3        | human prostate adenocarcinoma cell line       | RPMI    | ATCC     | R/R*               | II         |
| DUI145     | human prostate adenocarcinoma cell line       | RPMI    | ATCC     | G/G*               | II         |
| LnCap      | human prostate adenocarcinoma cell line       | RPMI    | ATCC     | R/G*               | II         |
| BT-474     | human breast carcinoma cell line              | RPMI    | ATCC     | G/G                | II, III    |
| BT-549     | human breast carcinoma cell line              | RPMI    | ATCC     | G/G**              | III        |
| MCF-7      | human breast carcinoma cell line              | RPMI    | ATCC     | G/G**              | III        |
| MDA-MB-231 | human breast carcinoma cell line              | RPMI    | ATCC     | G/G                | I, II, III |
| MDA-MB-453 | human breast carcinoma cell line              | RPMI    | ATCC     | R/R                | I, II, III |
| MDA-MB-468 | human breast carcinoma cell line              | RPMI    | ATCC     | G/G                | II         |
| Hs578T     | human breast carcinoma cell line              | RPMI    | ATCC     | R/R                | III        |
| SUM159     | human breast carcinoma cell line              | RPMI    | Asterand | R/G                | III        |
| T47D       | human breast carcinoma cell line              | RPMI    | ATCC     | R/G**              | III        |
| ZR-75-1    | human breast carcinoma cell line              | RPMI    | ATCC     | R/G                | II, III    |
| COLO205    | human colorectal carcinoma cell line          | RPMI    | ATCC     | R/G                | II         |
| HT-29      | human colorectal carcinoma cell line          | RPMI    | ATCC     | R/G                | II         |
| SW480      | human colorectal carcinoma cell line          | RPMI    | ATCC     | G/G                | II         |
| WiDr       | human colorectal carcinoma cell line          | RPMI    | ATCC     | R/G                | II         |

\*(Sahadevan et al., 2007) \*\*(Bange et al., 2002)

### 3 Antibodies, chemicals, and growth factors (I, II, III)

| Antibody:            | Description:   | Source:                  | Used in: |
|----------------------|--|--------------------------|----------|
| $\alpha$ -tubulin    | fabbit polyclonal antibody against $\alpha$ -tubulin     | SIGMA                    | III      |
| $\beta$ -tubulin     | rabbit polyclonal antibody against $\beta$ -tubulin      | Santa-Cruz Biotechnology | I, II    |
| Cadherin-11          | mouse monoclonal antibody against cadherin-11            | Invitrogen               | II       |
| CD44                 | mouse monoclonal antibody against CD44                   | Santa-Cruz Biotechnology | III      |
| CDC42                | mouse monoclonal antibody against CDC42                  | Santa-Cruz Biotechnology | III      |
| Clathrin heavy chain | mouse monoclonal antibody anti clathrin heavy chain      | Affinity Bioreagent      | I        |
| Collagen I           | rabbit polyclonal antibody against mouse collagen type I | Chemicon                 | II       |
| Collagen IV          | rabbit polyclonal antibody against collagen type I       | Chemicon                 | II       |
| Collagen IV          | goat polyclonal antibody against collagen type I         | Chemicon                 | II       |
| E-Cadherin           | mouse monoclonal antibody against E-cadherin             | BD Bioscience            | II       |
| EEA1                 | mouse monoclonal antibody against EEA1                   | BD Bioscience            | I        |
| EphA2 (C-terminal)   | rabbit polyclonal antibody against EphA2 (C-terminal)    | Santa-Cruz Biotechnology | III      |
| EphA2 (N-terminal)   | goat polyclonal antibody against EphA2 (N-terminal)      | R&D systems              | III      |
| ephrinA1             | rabbit polyclonal antibody against ephrinA1              | Santa-Cruz Biotechnology | III      |
| FGFR1                | goat polyclonal antibody against FGFR1                   | Abcam                    | I        |
| FGFR1                | rabbit polyclonal antibody against FGFR1                 | Santa-Cruz Biotechnology | I        |
| FGFR2                | rabbit polyclonal antibody against FGFR2                 | Santa-Cruz Biotechnology | I        |
| FGFR4 (C-terminal)   | rabbit polyclonal antibody against FGFR4 (C-terminal)    | Santa-Cruz Biotechnology | I        |
| FGFR4 (N-terminal)   | rabbit polyclonal antibody against FGFR4 (N-terminal)    | Santa-Cruz Biotechnology | I        |
| GFP                  | mouse monoclonal antibody against GFP                    | Dr. Emmy                 | III      |



|  |   | Verschuren               |            |
|--|---|--------------------------|------------|
| GAPDH                                  | mouse monoclonal antibody against GAPDH   | Sigma                    | III        |
| HA.11 (16B12)                          | mouse monoclonal antibody against HA  | Covance                  | I, II      |
| HA (6E2)                               | mouse monoclonal antibody against HA  | Cell signaling           | III        |
| IRAK1                                  | rabbit polyclonal antibody against IRAK1  | Santa-Cruz Biotechnology | I          |
| MT1-MMP                                | rabbit polyclonal antibody against MT1-MMP  | (Lehti et al., 2000)     | I, II, III |
| MT1-MMP                                | mouse monoclonal antibody against MT1-MMP   | (Ingvarsen et al., 2008) | II         |
| MT1-MMP (hinge domain)                 | rabbit polyclonal antibody against MT1-MMP  | Chemicon                 | III        |
| MT1-MMP (LEM-2/15.8, catalytic domain) | mouse monoclonal antibody against MT1-MMP   | Chemicon                 | III        |
| N-Cadherin                             | mouse monoclonal antibody against N-cadherin  | BD Bioscience            | II         |
| pAkt                                   | rabbit polyclonal antibody against phosphoAkt   | Cell Signaling           | II         |
| pan-Cadherin                           | rabbit polyclonal antibody against highly conserved sequence from the C-terminal of Cadherins | Sigma                    | II         |
| pERK                                   | rabbit polyclonal antibody against phosphoERK   | Cell Signaling           | II         |
| pFRS2                                  | rabbit polyclonal antibody against phosphoFRS2  | Cell Signaling           | II         |
| pPLC $\gamma$                          | rabbit polyclonal antibody against phosphoPLC $\gamma$  | Cell Signaling           | II         |
| pSrc                                   | rabbit polyclonal antibody against phosphoSrc kinase family                                   | Cell Signaling           | I, III     |
| pY                                     | mouse monoclonal antibody against phosphoTyrosine   | Cell Signaling           | I, II, III |
| Rac1                                   | mouse monoclonal antibody against Rac1  | Santa-Cruz Biotechnology | III        |
| RhoA                                   | mouse monoclonal antibody against RhoA  | Santa-Cruz Biotechnology | III        |
| Src                                    | rabbit polyclonal antibody against Src  | Cell Signaling           | I, III     |
| Trans-golgi network (Golgin97)         | mouse monoclonal antibody against Golgin97  | Invitrogen               | III        |
| Tubulin                                | mouse monoclonal antibody against Tubulin   | Sigma                    | III        |
| V5                                     | mouse monoclonal antibody against V5-tag  | Invitrogen               | I, III     |
| vWF                                    | Rabbit polyclonal antibody against vWF  | DAKO                     | II         |
| ZO-1                                   | Rabbit polyclonal antibody against ZO-1   | Zymed                    | II         |
| Mouse IgG                              | Mouse antibody isotype  | DAKO                     | II, III    |
| Rabbit IgG                             | Rabbit antibody isotype   | DAKO                     | II, III    |

#### 4 Chemicals and growth factors

| Chemicals or growth factors: | Description:   | Working concentration: | Source:           | Used in:   |
|------------------------------|--|------------------------|-------------------|------------|
| PMA                          | phorbol 12-myristate   | 4 nM or 40 nM          | Sigma             | I          |
| Bafilomycin A                | lysosomal inhibitor  | 100 nM                 | Calbiochem        | I          |
| GM6004                       | MMP inhibitor  | 10 $\mu$ M             | Calbiochem        | I, III     |
| MG132                        | proteasomal inhibitor  | 5 $\mu$ M              | Peptide Institute | I          |
| PP2                          | Src family kinase inhibitor  | 5 $\mu$ M              | Calbiochem        | I          |
| G418                         | selection reagent for stably plasmid DNA expressing cells                    | 400 $\mu$ g/mL         | Calbiochem        | I          |
| Puromycine                   | selection reagent for lentiviral infected stable cells                       | 2 $\mu$ g/mL           | SIGMA             | II, III    |
| ephrinA1-Fc                  | Recombinant mouse ephrinA1 extracellular domain/Fc chimera, ligand for EphAs | 1 mg/mL                | SIGMA             | III        |
| FGF2-Fc                      | Recombinant human FGF2/Fc chimera, ligand for FGFRs                          | 25 ng/mL               | Chemicon          | I, II      |
| HGF                          | hepatocyte growth factor   | 1 mg/mL                | SIGMA             | I, II, III |
| Dynasore                     | Dynamine inhibitor   | 80 mg/ml               | Sigma             | III        |

## 5 DNA and transfection (I, II, III)

Cells were transfected with expression vectors (listed below) using FuGENE HD (Roche). The lentiviral expression vectors were co-transfected with the packaging plasmid (pCMVdr8.74) and the envelope plasmid (pMD2-VSVG) to 293FT producer cells using lipofectamine 2000 (Invitrogen). The lentiviral Renilla luciferase–green fluorescent protein (GFP) fusion reporter protein expression vectors were co-transfected with the packaging and the envelope plasmids to 293-GPG producer cells.

| Name:                               | Vector:   | Insert:  | Tag:   | Source:                  | Used in: |
|-------------------------------------|---|--|--------|--------------------------|----------|
| <b>Mammalian expression vectors</b> |   |  |        |                          |          |
| pCR3.1                              | empty vector with neomycin resistance (CMV promoter)                        |  |        | Invitrogen               | I, III   |
| pCR3.1-MT1-MMP                      | pCR3.1  | encoding full length of MT1-MMP  |        | (Lehti et al., 2000)     | I, III   |
| pCR3.1-MT1-E/A                      | pCR3.1  | encoding full length of MT1-MMP with E240A mutation                                    |        | (Lehti et al., 2000)     | I, III   |
| pCR3.1-MT1-ΔCat                     | pCR3.1  | encoding pro and catalytic domains deleted MT1-MMP                                     |        | (Lehti et al., 2002)     | III      |
| pCR3.1-MT1-ΔCyt                     | pCR3.1  | encoding cytoplasmic tail deleted MT1-MMP  |        | (Lehti et al., 2002)     | I, III   |
| pCR3.1-MT1-Y/F                      | pCR3.1  | encoding full length of MT1-MMP with Y573F mutation                                    |        |                          | I        |
| pCR3.1 FGFR4-G388                   | pCR3.1  | encoding full length of FGFR4-G388   |        | Dr. Kari Alitalo         | I        |
| pCR3.1 FGFR4-G388-ΔC                | pCR3.1  | encoding cytoplasmic tail deleted FGFR4-G388   |        | Dr. Kari Alitalo         | I        |
| pCR3.1 FGFR4-G388-ΔK                | pCR3.1  | encoding kinase domain deleted FGFR4-G388  |        | Dr. Kari Alitalo         | I        |
| MT1-HA                              |   | encoding full length of MT1-MMP with HA-tag within the hinge domain                    | HA     | Dr. Stephen J Weiss      | I, III   |
| MT1-E/A-HA                          |   | encoding full length of MT1-MMP with E240A mutation and HA-tag within the hinge domain | HA     | Dr. Stephen J Weiss      | I        |
| MT1-Y/F-HA                          |   | encoding full length of MT1-MMP with Y573F mutation and HA-tag within the hinge domain | HA     |                          | I        |
| MT2-HA                              |   | encoding full length of MT2-MMP with HA-tag within the hinge domain                    | HA     | Dr. Stephen J Weiss      | III      |
| MT3-HA                              |   | encoding full length of MT3-MMP with HA-tag within the hinge domain                    | HA     | Dr. Stephen J Weiss      | III      |
| pCMV6-XL5                           | empty vector with neomycin resistance (CMV promoter)                        |  |        | Origene                  | I        |
| pCMV6-XL5-IRAK1                     | pCMV6-XL5   | encoding full length of IRAK1 (NM 001569)  |        | (Varjosalo et al., 2008) | I        |
| pCMV6-XL5-FGFR1 III                 | pCMV6-XL5   | encoding full length of FGFR1 III (NM 023110)  |        | (Varjosalo et al., 2008) | I        |
| pCMV6-XL5-FGFR1 IV                  | pCMV6-XL5   | encoding full length of FGFR1 IV (NM 015850)   |        | (Varjosalo et al., 2008) | I        |
| pCMV6-XL5-FGFR2 IIIc                | pCMV6-XL5   | encoding full length of FGFR2 IIIc (NM 001144915)                                      |        | (Varjosalo et al., 2008) | I        |
| pCMV6-XL5-FGFR3 IIIc                | pCMV6-XL5   | encoding full length of FGFR3 IIIc (NM 000142)   |        | (Varjosalo et al., 2008) | I        |
| pCMV6-XL5-FGFR4-R388                | pCMV6-XL5   | encoding full length of FGFR4 R388 (NM 002011)   |        | (Varjosalo et al., 2008) | I        |
| pcDNA3.1/V5-HisC                    | empty vector with V5 and His tagging and neomycin resistance (CMV promoter) |  |        | Invitrogen               |          |
| pcDNA3.1/V5-HisC FGFR4-G388         | pcDNA3.1/V5   | encoding full length of FGFR4-G388   | V5-His | Dr. Jussi Taipale        | I, II    |
| pcDNA3.1/V5-HisC FGFR4-G388-KD      | pcDNA3.1/V5   | encoding full length of FGFR4-G388 with K503M mutation                                 | V5-His | Dr. Jussi Taipale        | I        |

|                                   |  |   |        |                      |         |
|-----------------------------------|--|---|--------|----------------------|---------|
| pcDNA3.1/V5-HisC<br>FGFR4-R388    | pcDNA3.1<br>/V5  | encoding full length of FGFR4-<br>R388  | V5-His | Dr. Jussi<br>Taipale | I, II   |
| pcDNA3.1/V5-HisC<br>FGFR4-R388-KD | pcDNA3.1<br>/V5  | encoding full length of FGFR4-<br>R388 with K503M mutation                              | V5-His | Dr. Jussi<br>Taipale | I       |
| pcDNA3.1/V5-HisC<br>EphA2         | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>(NM 004431)  | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-D/I     | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with D358I mutation                                    | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-G/I     | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with G391I mutation                                    | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-G/R     | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with G391R mutation                                    | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-DIV/AAA | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with DIV359-361AAA<br>mutation                         | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-YSV/AAA | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with YSV362-364AAA<br>mutation                         | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-HGL/AAA | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with HGL390-392AAA<br>mutation                         | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-GLT/AAA | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with GLT391-393AAA<br>mutation                         | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-KD      | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with K646M mutation                                    | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-KD-D/I  | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with D358I and K646M<br>mutations                      | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-KD-G/I  | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with G391I and K646M<br>mutations                      | V5-His |                      | III     |
| pcDNA3.1/V5-HisC<br>EphA2-KD-G/R  | pcDNA3.1<br>/V5  | encoding full length of EphA2<br>with G391R and K646M<br>mutations                      | V5-His |                      | III     |
| <b>Lentiviral vectors</b>         |  |   |        |                      |         |
| pLVX-puro                         | empty vector with puromycin resistance<br>(CMV promoter) |   |        | Clontech             |         |
| pLVX-EphA2-V5-His                 | pLVX-<br>puro  | full length of EphA2 with C-<br>terminus V5 and His tag                                 | V5-His |                      | III     |
| pLVX-EphA2-D/I-V5-His             | pLVX-<br>puro  | full length of EphA2 with<br>D358I mutation and C-terminus<br>V5 and His tag            | V5-His |                      | III     |
| pLVX-EphA2-G/I-V5-His             | pLVX-<br>puro  | full length of EphA2 with<br>G391I mutation and C-terminus<br>V5 and His tag            | V5-His |                      | III     |
| pLVX-EphA2-KD-V5-His              | pLVX-<br>puro  | full length of EphA2 with<br>K646M mutation and C-<br>terminus V5 and His tag           | V5-His |                      | III     |
| pLVX-EphA2-KD-D/I-<br>V5-His      | pLVX-<br>puro  | full length of EphA2 with<br>D358I and K646M mutations<br>and C-terminus V5 and His tag | V5-His |                      | III     |
| pLVX-EphA2-KD-G/I-<br>V5-His      | pLVX-<br>puro  | full length of EphA2 with<br>G391I and K646M mutations<br>and C-terminus V5 and His tag | V5-His |                      | III     |
| <b>Retroviral vectors</b>         |  |   |        |                      |         |
| GFP-Renilla Luciferase            |  | encoding GFP and renilla<br>luciferase  | GFP    | Dr. Kari<br>Alitalo  | II, III |

## 6 cDNA mutagenesis assay (I, III)

HA-tagged MT1-E/A and MT1-Y/F, FGFR4-G388, and EphA2 mutant cDNAs were generated by PCR mediated overlap extension method with site-directed-mutagenesis (Stratagene) using primers listed below.

| Name:         | Forward primer:                                  | Reverse primer:                                  | Used in: |
|---------------|--|--|----------|
| MT1-MMP-E/A   | GTG GCT GTG CAC GCG CTG GGC CAT GCC C            | G GGC ATG GCC CAG CGC GTG CAC AGC CAC            | I        |
| MT1-MMP-Y/F   | C AGG CGA CTG CTC TTC TGC CAG CGT TCC C          | G GGA ACG CTG GCA GAA GAG CAG TCG CCT G          | I        |
| FGFR4-G388    | CTG CTG CTG GCC GGG CTG TAT CGA GGG              | CCC TCG ATA CAG CCC GGC CAG CAG CAG              | I, II    |
| EphA2-D/I     | C CGG GGC CGC GAG ATC ATT GTC TAC AGC G          | C GCT GTA GAC AAT GAT CTC GCG GCC CCG G          | III      |
| EphA2-G/I     | G GAG CCT CCT CAC ATA CTG ACC CGC ACC AG         | CT GGT GCG GGT CAG TAT GTG AGG AGG CTC C         | III      |
| EphA2-G/R     | GAG CCT CCT CAC CGA CTG ACC CGC ACC AG           | CT GGT GCG GGT CAG TCG GTG AGG AGG CTC           | III      |
| EphA2-DIV/AAA | GG GGC CGC GAG GCC GCT GCC TAC AGC GTC ACC       | GGT GAC GCT GTA GGC AGC GGC CTC GCG GCC CC       | III      |
| EphA2-YSV/AAA | C GTC ACC TGC GAA CAG GCC GCG GCC GAG TCT GGG G  | C CCC AGA CTC GGC CGC GGC CTG TTC GCA GGT GAC G  | III      |
| EphA2-HGL/AAA | C TCG GAG CCT CCT GCC GCA GCG ACC CGC ACC AGT G  | C ACT GGT GCG GGT CGC TGC GGC AGG AGG CTC CGA G  | III      |
| EphA2-GLT/AAA | G GAG CCT CCT CAC GCA GCG GCC CGC ACC AGT GTG AC | GT CAC ACT GGT GCG GGC CGC TGC GTG AGG AGG CTC C | III      |

## 7 siRNAs and shRNAs (I, II, III)

siRNA and shRNAs used in this study are listed below. siRNAs were transfected using Lipofectamine 2000 (Invitrogen). shRNAs were used to produce lentiviral particles and the infected cells were followed by selection with puromycine. Knockdown efficiency was monitored by quantitative RT-PCR after 48 h.

| siRNA:               | Source: | Used in:   | shRNA:                   | Source:         | Used in:   |
|----------------------|---------|------------|--------------------------|-----------------|------------|
| non-targeting        | Qiagen  | I, II, III | non-targeting scrambled  | Open Biosystems | I, II, III |
| MT1-MMP (SI03648841) | Qiagen  | I, II, III | MT1-MMP (TRCN0000050855) | Open Biosystems | I, II, III |
| FGFR1 (SI02224677)   | Qiagen  | II         | MT1-MMP (TRCN0000050853) | Open Biosystems | I, II      |
| FGFR1 (SI03059252)   | Qiagen  | II         | FGFR4 (TRCN0000000628)   | Open Biosystems | II         |
| FGFR4 (SI02665306)   | Qiagen  | II         | FGFR4 (TRCN0000010531)   | Open Biosystems | II         |
| EphA2 (SI00031360)   | Qiagen  | III        | EphA (TRCN0000006403)    | Open Biosystems | III        |
| EphA2 (SI00031360)   | Qiagen  | III        | EphA2 (TRCN0000006405)   | Open Biosystems | III        |
| EphA2 (SI00031360)   | Qiagen  | III        | EphA2 (TRCN0000006404)   | Open Biosystems | III        |
| EphA2 (SI00031360)   | Qiagen  | III        | EphA2 (TRCN0000006406)   | Open Biosystems | III        |
| EphA2 (SI00031360)   | Qiagen  | III        | EphA2 (TRCN0000006407)   | Open Biosystems | III        |

## 8 RNA extraction and quantitative real-time PCR (I, II, III)

mRNA was extracted from the cultured cell lines, human skin samples, and mouse tissues with RNeasy Mini Kit (Qiagen) followed by reverse transcription with iScript reverse transcriptase (Life Technologies) according to manufactures' instructions. mRNA expression

was quantified using TaqMan Universal PCR Master Mix and validated Taqman® primers (MT1-MMP, Hs 01037006\_gH; FGFR4, Hs00242558\_m1; EphA2, Hs00171656\_m1, ephrinA1, Hs00358886\_m1; Applied Biosystems). The expression was normalized with TATA-binding protein (TBP) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA expression.

## **9 Gelatine zymography (I)**

Cells were cultured in complete medium for 20 h in serum-free medium. The conditioned media were collected and polypeptides in the medium were separated under non-reducing conditions using 10% SDS-polyacrylamide gels containing 1 mg/ml of gelatin. The gels were washed twice with 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM CaCl<sub>2</sub>, 1 μM ZnCl<sub>2</sub> and 2.5% Triton X-100 for 15 min to remove SDS, followed by a brief rinsing in washing buffer without Triton X-100. Next, the gels were incubated at 37 °C for 16 hours in the developing buffer, 50 mM Tris-HCl, pH 7.6, containing 5 mM CaCl<sub>2</sub>, 1 μM ZnCl<sub>2</sub>, 1% Triton X-100 and 0.02% NaNO<sub>3</sub>. The gels were then stained with Coomassie Brilliant Blue for 2 h and destained with a solution containing 10% methanol and 10% acetic acid.

## **10 Immunoblotting, immunoprecipitation, and mass spectrometry analyses (I, II, III)**

Cell lysates were prepared using RIPA lysis buffer (50 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% NaDOC, 0.1% SDS, and 0.02% (w/v) sodium azide, complete protease inhibitor cocktail (Roche), and 10 μM of GM6001) and insoluble materials were removed by centrifugation. For immunoprecipitation, two times volume of Triton lysis buffer (50 mM Tris-HCl buffer, pH 8.0, containing 150 mM NaCl, 1% (v/v) Triton X-100, 0.02% (w/v) sodium azide, complete protease inhibitor cocktail, and 10 μM GM6001) was added to each soluble cell lysate, followed by incubation with antibody conjugated agarose beads. After washes with the Triton lysis buffer, the protein complexes were eluted with Laemmli SDS-PAGE sample buffer. The soluble cell lysates and immunoprecipitated proteins were resolved by SDS-PAGE with a linear 4–20% gradient gel under reducing conditions followed by immunoblotting. For mass spectrometry analysis, immunoprecipitated proteins were subjected to SDS-PAGE followed by silver staining using Proteosilver Plus Stain Kit (Sigma). The aimed bands were cut out from the silver stained gels and subjected to mass spectrometry analysis (LC-MS/MS) after trypsin digestion.

## **11 Cell surface biotinylation**

Cells were rinsed at 4°C with ice-cold PBS and incubated in PBS containing 0.5 mg/ml of Sulfo-NHS-biotin (Thermo Fisher Scientific) on ice for 1 h. The reaction was terminated by washing with 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM glycine for 10 min. The soluble cell lysates were subjected to immunoprecipitation and the immune complexes were detected with horseradish peroxidase-conjugated streptavidin (Dako).

## **12 Immunofluorescence (I, II, III)**

Cells grown on collagen coated glass coverslips were washed with PBS and fixed with 4% PFA. After washing with Dulbecco's PBS, the cells were incubated in Dulbecco's PBS containing 5% BSA and then incubated with the primary antibody for 1 h. The bound antibodies were detected using Alexa Fluor-conjugated secondary antibodies (Invitrogen). The coverslips were mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories). The cells were photographed by confocal imaging using LSM 5 DUO or LSM 780 confocal microscopes (Carl Zeiss).

## **13 3D type I collagen invasion and invasive growth analyses (I, II, III)**

Rat tail type I collagen (Sigma) was dissolved in 0.2% acetic acid and neutralized with NaOH and diluted to a final concentration of 2.2 mg/ml in MEM. The collagen solution was cast 150  $\mu$ l into the upper chamber of Falcon cell culture inserts in 24-well cell culture plates and allowed to form a gel at 37°C for 1 hour. Tumour cells ( $3 \times 10^5$ /insert) in completed culture media were added on top of the gel. The medium was supplemented with recombinant human HGF and FGF2 as chemoattractants to the lower chamber. The cells were cultured at 37°C for 5 days replacing them with complete medium every second/third day. The gels were then fixed in 4% PFA, dehydrated and embedded into paraffin. Invading cells were counted and photographed from hematoxylin and eosin (H&E) stained paraffin sections using a Leica DM LB microscope and Image J software.

Single cell suspension ( $3 \times 10^3$  cells/gel) was prepared in collagen I solution and formed collagen gel drops in 24-well cell culture plates. The gels were cultured for 4-6 days in completed culture media. The colonies were stained after 4% PFA fixation with TRITC-conjugated phalloidin (Sigma) or indirect immunofluorescence staining and photographed using an LSM 5 DUO confocal microscope (Carl Zeiss).

## **14 Mouse tumour growth analysis (II, III)**

Cells were lentivirally transduced with a Renilla luciferase-GFP fusion reporter protein. The cells ( $2 \times 10^6$  cells/mouse) were implanted orthotopically into the abdominal subcutis of Severe combined immunodeficient (SCID) mice (5-7 weeks of age; Taconic) and allowed to grow for 4 to 8 weeks. Tumour growth was measured by a calliper, and metastasis to local lymph nodes were followed by non-invasive bioluminescence measurement after i.p. injection of coelenterazine (35  $\mu$ g in 100  $\mu$ L PBS; Synchem) using a Xenogen IVIS System.

## **15 Histologic analyses and immunohistochemistry (II, III)**

Mouse tumours and lymph nodes were collected and fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin followed by H&E, Herovici, and immunohistochemistry staining. The tissue sections were de-paraffinized in TissueClear (Tissue-Tek) and re-hydrated in graded ethanol series. After antigen retrieval by boiling in

sodium citrate buffer (10 mM Na citrate, 0.05% Tween-20, pH 6), the sections were incubated for 10 min in 0.6% (v/v) hydrogen peroxide. They were subsequently rinsed and incubated with 2.5% Normal Horse Serum (Vector Laboratories) for 30 min. Primary antibodies were incubated for 2 hours in blocking buffer, followed by incubation with peroxidase conjugated anti-mouse Ig/anti-rabbit Ig for 30 min. Detection was performed using Vectastain ABC kit according to manufacturer's instructions. After washes slides were dehydrated and mounted using Cytoseal mounting medium (Electron Microscopy Sciences). The mouse tissues were also subjected to frozen sectioning (20  $\mu$ m) followed by immunofluorescence staining. Vessels with intravasated tumor cells were counted from H&E-stained sections.

Frozen or paraffin embedded human multiple human tumour and normal tissue arrays (FMC401, BR401, and BRM961; US Biomax, Inc.) were subjected to immunohistochemistry with pre-titrated dilutions of antibodies.

## **16 FGFR4 allele genotyping analysis (I, II)**

The fragments of FGFR4 cDNA containing the G388R site (1,329–1,331 bp) were amplified by PCR (primers: TACCAGTCTGCCTGGCTC and AGTACGTGCAGAGGCCTT) and digested with BstN1 (New England Biolabs). R388 allele was identified by a specific 126-bp fragment, and G388 allele by two fragments (97 and 29 bp). Unclear results were confirmed by sequencing.

## **17 Rho-GTPase activity assay (III)**

Soluble cell lysates were incubated with Rhotekin RBD-GST (for the RhoA activity assay) or PAK-1 PDB-conjugated agarose beads (for Rac1 and CDC42 activity assay) for 4 h at 4°C. The resulting bound Rho-GTPase complexes were analyzed by immunoblotting. Total RhoA, Rac1, and Cdc42 were detected from soluble cell lysates.

## **18 Cell detachment and cell-cell repulsion analysis (III)**

Time-lapse microscopy imaging was performed on an Intelligent Imaging Innovations (3i) - Stallion HIS (Zeiss) microscope with a Zeiss AxioCam MRm camera and SlideBook 4.1 software. The cells were seeded on chambers (BD) coated with monomeric collagen I ( $1.0 \times 10^5$  cells/cm<sup>2</sup>). An image was taken every 5 min at 37 °C with 5% CO<sub>2</sub> for up to 5 or 12 h. All movies were compiled and cell tracking was done using ImageJ MTrackJ and Manual Tracking Plug-In. The cell detachment or attachment responses were determined by counting the number of detaching or attaching cells followed for 90 min after cell collision. Quantification of cell repulsion was carried out as previously described (Paddock and Dunn, 1986). In brief, the displacements 35 minutes before collision (A) and for 35 min following collision (B) were measured. The contact acceleration index (Cx) of vector B–A represents the difference between how far the cell has progressed in the direction of A and how far it would have gone had there been no collision. The velocity of each cell was determined by

tracking the position of the nucleus in the first and last images divided by the total time and represented in a compass graph using MathLab software.

## **19 Statistical analysis (I, II, III)**

All numerical values represent mean +/- SEM. Statistical significance was determined using two-tail Student's t-tests or Mann-Whitney U test.



## RESULTS AND DISCUSSION

### 1 Identification of receptor tyrosine kinases, FGFR4 and EphA2, as unique regulators of proinvasive MT1-MMP activity (I, II, III)

Many types of tumour cells up-regulate MT1-MMP and use its proteolytic activities for their invasion. MT1-MMP proteolytic activities are regulated at the transcriptional or the posttranscriptional levels by several growth factor/cytokine-induced signalling and through interaction with cell surface or intracellular signalling molecules. However, cancer-associated molecular mechanisms of pro-invasive MT1-MMP activities have not been fully defined.

To identify the upstream and co-operative protein kinase signalling for pro-invasive MT1-MMP activity in cancer cells, we set up a systematic genome-wide gain-of-function human kinome screen. Individual 564 cDNAs constituting ~93% of all human protein kinases (Varjosalo et al., 2008) were expressed in human HT-1080 fibrosarcoma cells. The levels of proMMP2 activation were quantified as an indirect indication for MT1-MMP activities. From the primary screen, we chose the top 32 kinases for second screen (Fig. 1A in I). Among them, 21 kinases increased MMP-2 activation more than 2-fold relative to the mock-transfected control (Fig. 1D in I). These kinases contained both novel and known MT1-MMP positive regulators. The latter group included downstream signalling molecules of IL-1 and TNF- $\alpha$  signalling as well as receptors of TGF- $\beta$  signalling (Fig. 1D in I). These signalling pathways are associated with MT1-MMP transcription (Lohi et al., 1996; Munshi et al., 2004; Rajavashisth et al., 1999), which thus validated the kinome screen. The novel MT1-MMP positive regulators involved two RTKs, FGFR4-R388 variant and EphA2. This library also contained other three FGFR family members (FGFR1, FGFR2, and FGFR3) and ten Eph receptors (EphA1, EphA3, EphA4, EphA5, EphA7, EphB1, EphB2, EphB3, EphB4, and EphB6). However, none of these kinases individually increased efficiently MT1-MMP-mediated MMP2 activation over 2-fold (Fig. S1C in I, Fig.1A in III). We continued to study the cooperative molecular mechanisms of pro-invasive MT1-MMP and FGFR4-R388 or EphA2 in cancer cell invasion, since the cell surface receptor kinases can be potential therapeutic targets for patients with cancer.

### 2 FGFR4 polymorphism acts as an activity switch of MT1-MMP-mediated cancer cell invasion (I, II)

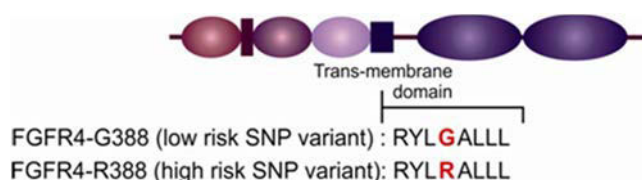


Figure 19. Structure of the FGFR4 variants. FGFR4-R388 SNP is associated with aggressive cancer progression and poor prognosis of patients with several types of cancer.

Although MT1-MMP can modulate FGFR2 signalling through MT1-MMP-FGFR2-ADAM9 interaction in mouse calvarial osteogenesis (Chan et al., 2012), cancer-associated molecular mechanisms that are linking FGFRs to pro-invasive MT1-MMP activities have not been

reported. The FGFR4-R388 polymorphism has Gly to Arg conversion in its transmembrane domain (Figure 19). This variant expression has been linked to aggressive cancer progression and poor prognosis of patients with several types of tumours (Bange et al., 2002; da Costa Andrade et al., 2007; Spinola et al., 2005; Streit et al., 2004). However, the underlying molecular mechanisms of increased cancer progression caused by the FGFR4-R388 risk variant expression have remained unclear.

## **2.1 The FGFR4-R388 risk variant increases MT1-MMP levels by reducing lysosomal degradation of MT1-MMP (I)**

Since *MT1-MMP* gene expression is frequently up-regulated in malignant conditions, we first examined the effect of the FGFR4-R388 risk variant on *MT1-MMP* transcript. The FGFR4-R388 did not affect *MT1-MMP* mRNA expression in HT-1080 cells and human breast carcinoma MDA-MB-231 cells (Fig. S2A in I). This variant was thought to regulate MT1-MMP activity at posttranscriptional levels. Cell surface MT1-MMP is regulated by endocytosis coupled with lysosomal degradation or recycling to the cell surface (Zucker et al., 2002). We tested whether the FGFR4-R388 risk variant inhibits MT1-MMP degradation. In MDA-MB-231 cells, lysosomal inhibitor Bafilomycin A markedly increased endogenous MT1-MMP, while a proteasome inhibitor MG134 did not affect MT1-MMP levels (Fig. 2A in I). Consistent with an increased MT1-MMP activity, the FGFR4-R388 variant increased MT1-MMP protein level. In this variant expressing cells, the effect of Bafilomycin A was minor on MT1-MMP level (Fig. 2A-C in I). Therefore, lysosomal degradation of MT1-MMP is inhibited in the FGFR4-R388 risk variant expressing cells. In contrast, the FGFR4-G388 variant expression decreased MT1-MMP levels (Fig. 2C and D in I). Importantly, this variant was up-regulated by inhibition of MT1-MMP activity and down-regulated by the increased MT1-MMP after Bafilomycin A treatment (Fig. 2C in I). Although MT1-MMP was co-precipitated with these two FGFR4 variants as well as their corresponding kinase activity-deficient (KD) proteins (Fig. 2D, S3A-B in I), the catalytically active FGFR4-R388 and FGFR4-G388 variants showed different effects on MT1-MMP protein stability.

## **2.2 The FGFR4-R388 risk variants increases MT1-MMP phosphorylation and endosomal stabilization (I)**

We next studied how the FGFR4 variants regulate MT1-MMP stability. In stable FGFR4-R388-expressing MDA-MB-231 cells, ectopic FGF2 treatment increased the phosphorylation levels of both FGFR4 and MT1-MMP, interaction of these proteins, and accumulation in the same endosomal compartments (Fig. 3A-D in I). In contrast, the co-localization of MT1-MMP and the FGFR4-G388 or the KD proteins was poor and activated low risk variant did not increase MT1-MMP phosphorylation (Fig. 3A-C and S6 in I). Phosphorylation of a single tyrosine residue in the cytoplasmic tail of MT1-MMP by non-receptor tyrosine kinase Src regulates MT1-MMP endocytosis, which is also associated with tumour growth and invasion (Moss et al., 2009; Nyalendo et al., 2007; Nyalendo et al., 2008). Interestingly, MT1-MMP phosphorylation by the FGFR4-R388 variant was inhibited by Src inhibitor, PP2 (Fig. S4D in I). Src is a downstream mediator of FGF signalling. The FGFR4-R388 risk variant-induced signalling thus can increase MT1-MMP phosphorylation and stability of both FGFR4-R388 and MT1-MMP in the same endosomal vesicles through intracellular Src activity.

We further examined the stability of the FGFR4 variants themselves after ligand stimulation. The FGFR4-R388 risk variant was more stable compared to the FGFR4-G388 low risk variant after FGF2 treatment (Fig. 3E in I). The FGFR4-G388 variant was degraded rapidly after ligand-induced activation, which could be involved in MT1-MMP proteolytic activity since a synthetic broad-spectrum MMP inhibitor GM 6001 treatment sustained the expression levels of this low risk variant (Fig. 3E in I). Correlated with the stability of these variants, MT1-MMP levels were increased after ligand stimulation only in the FGFR4-R388 variant expressing cells (Fig. 3E in I). The increased stability of the FGFR4-R388 variant rather than the physical interactions of FGFR-MT1-MMP can thus enhance the stabilization of the phosphorylated and endocytosed MT1-MMP. The increased stability of the activated FGFR4-R388 sustains its autophosphorylation (Wang et al., 2008). Furthermore, an analogous of achondroplasia-causing mutation in FGFR3 transmembrane domain (FGFR3-G380R) leads to increased kinase activity and stability of the receptor recycling and decreased targeting of receptors to lysosomes (Cho et al., 2004; Eswarakumar et al., 2005). Therefore, the FGFR4-R388 can alter the interactions of the activated receptor with vesicular sorting proteins for recycling instead of lysosomal degradation similarly to FGFR3 (Cho et al., 2004). MT1-MMP can be also sorted to the recycling pathways after endocytosis together with the FGFR4-R388 variant, where the activated FGFR4-R388 signalling increases MT1-MMP phosphorylation by Src. In contrast, the unstable FGFR4-G388-MT1-MMP complex is rapidly degraded after endocytosis. In this complex, proteolytic activity of MT1-MMP can be involved in this process.

### **2.3 MT1-MMP and FGFR4-R388 activate and MT1-MMP and FGFR4-G388 suppress each other (I)**

To study the impact of MT1-MMP phosphorylation on the interaction of the FGFR4 and MT1-MMP, we constructed the MT1-Y573F mutant in which the tyrosine residue in cytoplasmic domain was changed to phenylalanine. This mutant did not alter MT1-MMP-mediated MMP2 activation (Fig. S7B in I). In the endogenous FGFR4-R388 variant expressing MDA-MB-453 breast carcinoma cells, the wild-type MT1-MMP was co-localized with the FGFR4-R388 at cell junctions and intracellular vesicles (Fig. 4A in I). Consistent with the previous report (Moss et al., 2009), the MT1-Y/F mutant was accumulated on the cell surface. In this mutant expressing cells the FGFR4-R388 localization was mainly detected in the intracellular vesicles (Fig. 4A in I). Furthermore, MT1-Y/F led to cell junctional disassembly and elongated cell morphology (Fig. 4A in I). Consistently, immunoblotting analysis showed that the interaction of FGFR4-R388 with MT1-Y/F was weaker than with MT1-MMP (Fig. 4C in I). Of note, this FGFR4-R388-MT1-MMP interaction enhanced the phosphorylation of both MT1-MMP and the FGFR4-R388 variant (Fig. 3A-C and 4C in I). On the other hand, in FGFR4-G388 stably expressing MDA-MB-231 cells MT1-MMP and FGFR4-G388 were expressed in different intracellular vesicles (Fig. 4B in I). The expression of MT1-Y/F dramatically decreased FGFR4-G388 expression in the same cells (Fig. 4B in I).

On the cell surface, FGFR4 cooperates with cell-adhesion receptors, such as N-cadherin and NCAM to regulate cell-cell and cell-matrix adhesion (Cavallaro et al., 2001; Francavilla et al., 2007; Lehembre et al., 2008; Nakamura et al., 2008). The cancer cells used in this study,

MDA-MB-453 cells express negligible levels of cadherins, such as E-Cadherin, N-Cadherin, Cadherin-11, and P-cadherin (Nieman et al., 1999), however, they instead express other cell-surface adhesion proteins, e.g. epithelial cell adhesion molecule (EpCAM), and MDA-MB-231 cells express cadherin-11 (Nieman et al., 1999; Osta et al., 2004; Prang et al., 2005). At cell junctions, endogenous FGFR4-R388 may cooperate with these cell surface molecules to maintain cell–cell adhesion. Loss of FGFR4-R388 from the cell surface and cell–cell junctions in MT1-Y/F expressing cells can thus lead to cell-junctional disassembly and induction of EMT-like cell phenotype transition. In parallel, accumulation of MT1-Y/F on the cell surface facilitates the FGFR4-G388 variant degradation. Therefore, MT1-Y/F can escape from downregulation by the FGFR4-G388 variant and sustain its proteolytic activities.

#### **2.4 The FGFR4-G388R risk variant promotes MT1-MMP-mediated collagen degradation (I, II)**

Since MT1-MMP is essential for collagen degradation (Hotary et al., 2000), we examined the effect of the FGFR4 variants on MT1-MMP proteolytic activities in 3D collagen invasion assay. The cross-linked collagen I matrix typifies the ECM of collagen-rich stroma (Egeblad et al., 2010; Sabeh et al., 2009). The cells were seeded atop 3D collagen and allowed to invade for 5 days. FGFR4-R388 significantly increased MDA-MB-231 cell invasion, whereas the FGFR4-G388 and their KD proteins did not affect MT1-MMP-mediate collagen degradation (Fig. 5A, B and S9 in I). We further used human prostate carcinoma cell lines for testing the functional relevance of the endogenous MT1-MMP and the FGFR4-R388 or the FGFR4-G388 variant. PC3 prostate carcinoma cells express both MT1-MMP and the FGFR4-R388 risk variant, and DU145 cells express MT1-MMP and the FGFR4-G388 low risk variant. PC3 cells invaded into collagen gels efficiently, which was inhibited by the FGFR4-R388 variant knockdown (Fig. 3 in II). On the other hand, DU145 displayed low level invasiveness (Fig. 3 in II). As expected, invasion of all these cells was enhanced by MT1-MMP over-expression and strongly impaired after MT1-MMP knockdown (Fig. 5A, B and S9 in I and Fig. S4D in II). Thus, the FGFR4-R388 variant and MT1-MMP cooperatively promote breast and prostate carcinoma cell invasion into 3D collagen matrices. Consistent with increased cancer cell invasion, on the thin layers of gelatin or cross-linked collagen, or within 3D collagen matrices the FGFR4-R388 specifically increased MT1-MMP levels at the leading edges of invading cells (Fig. 5C and D in I).

Rapid endocytosis of MT1-MMP and its accumulation at the invasive front can promote cancer cell invasion efficiently (Bravo-Cordero et al., 2007; Poincloux et al., 2009; Remacle et al., 2003; Uekita et al., 2001). By increasing MT1-MMP in invasive protrusions, the FGFR4-R388 risk variant co-operatively enhances pericellular ECM degradation and further promotes cancer cell invasion. Taken these results, we proposed a schematic model of the function of FGFR4-MT1-MMP complexes shown in Fig. 4D in I. The both FGFR4 variants form complex with MT1-MMP, however, the different stability of each variant increases or decreases MT1-MMP levels and activities. The FGFR4-R388 risk variant forms a complex with MT1-MMP on the cell membranes, where this variant can phosphorylate MT1-MMP in its cytoplasmic tail via Src activity. The internalized the FGFR4-R388-MT1-MMP complexes can be selectively sorted into recycling endosomes, which may reflect higher stability of both proteins and increase the accumulation of MT1-MMP and the FGFR4-R388

at the leading edge of invading cells. In this complex, MT1-MMP also increases phosphorylation levels of this risk variant. Thus, the FGFR4-R388-MT1-MMP axis can enhance MT1-MMP proteolytic activity and prolong FGF signalling, resulting in aggressive breast and prostate carcinoma cell invasion. In contrast, the complexes with the unstable FGFR4-G388 variant are mainly delivered to lysosomes and rapidly degraded. The cell-surface accumulated MT1-MMP can also degrade the FGFR4-G388 variant and cell surface adhesion molecules, leading to cell junctional dissociation and induction of more motile-type elongated cell morphology.

### **3 FGFR4 and MT1-MMP are co-expressed at invasive edge in human tumours (II).**

FGFR4 is frequently overexpressed in human epithelial carcinomas, such as breast, prostate and hepatocellular carcinomas (Ho et al., 2009; Jaakkola et al., 1993; Sahadevan et al., 2007). The overexpressed FGFR4 contributes to cancer progression and poor prognosis (Ho et al., 2009; Sahadevan et al., 2007). In breast tumours, the overexpression of FGFR4 can also be often linked to anti-cancer drug resistance (Meijer et al., 2008). We studied the FGFR4-MT1-MMP axis in human tumour progression using human carcinoma biopsies and Gene Sapience database.

#### **3.1 The FGFR4-R388 variant associates with MT1-MMP mRNA expression in high grade (Grade 3) breast carcinomas (II)**

To study whether the FGFR4 variants and MT1-MMP are co-expressed in human tumours, we used a qPCR array from 48 human breast cancer biopsies. Consistent with the previous report (Bange et al., 2002), 56% of samples had homozygous or heterozygous FGFR4-R388 risk variant expression (R/R, 8%; G/R, 48%) and 44% of samples had homozygous FGFR4-G388 low risk variant expression (G/G; Fig. 1A in II). All homozygous FGFR4-R388 samples were detected from the highest-grade tumours coupled with low level of FGFR4 and increased MT1-MMP mRNA expression, which can be consistent with the poor prognosis reported for patients with this allele (Bange et al., 2002; da Costa Andrade et al., 2007; Spinola et al., 2005; Streit et al., 2004). No significant correlation was observed between the expression levels of *FGFR4* and *MT1-MMP* mRNAs in individual breast tissue biopsies (Fig. 1A in II), suggesting that the FGFR4-R388 variant may indirectly contribute to MT1-MMP transcription in the carcinoma cells and/or stroma *in vivo* within high grade human breast tumours.

#### **3.2 MT1-MMP and FGFR4 are co-expressed in stroma/tumour border and in invasive front of breast, lung, colon, and prostate carcinomas (II)**

To analyse the expression of both FGFR4 and MT1-MMP in different stage of human breast tumours, we assessed the expression and localization of these proteins in 42 normal or malignant breast tissue samples by immunohistochemistry. FGFR4 was faintly stained in luminal epithelium in normal breast tissues, whereas MT1-MMP was undetectable (Fig. 1C in II; modestly positive stroma in 2/4). Consistent with the previous reports (Okada et al., 1995; Szabova et al., 2005; Thussbas et al., 2006), FGFR4 was strongly detected in

carcinoma cells (20/38) and MT1-MMP was detected in reactive stroma (carcinoma in situ 2/8; invasive ductal carcinoma, 17/28; invasive lobular carcinoma, 2/2), in myoepithelium in breast carcinoma in situ (7/8), and in poorly differentiated carcinoma cells of invasive ductal carcinomas (13/28; Fig. 1C and D in II). Importantly, both FGFR4 and MT1-MMP were expressed in the latter two types of cells in breast carcinomas (Fig. 1C and D in II). We further assessed the co-expression of FGFR4 and MT1-MMP in many types of human tumours using 14 different normal and malignant human tissue samples. MT1-MMP was up-regulated in tumour cells (9/14) and/or reactive stroma (9/14), whereas FGFR4 expression was mainly detected in the tumour cells (5/14; Table S1 in II). Particularly, co-localization of MT1-MMP and FGFR4 proteins were detected in the leading edge of collectively invading lung squamous cell carcinomas and colon adenocarcinomas (Fig. 2A in II). Thus, the co-expression of these proteins is restricted to the tumour/stroma border and the invasive front of many types of carcinomas, as well as to highly invasive undifferentiated breast carcinoma cells.

GeneSapiens database analysis (Kilpinen et al., 2008) revealed that similar to the breast tissue biopsies, in overall expression of *FGFR4* and *MT1-MMP* expression was low or not correlated to each other in individual tissue biopsies from different human tumour types, even though *FGFR4* and *MT1-MMP* were frequently up-regulated in same tumour types (Fig. 1A and 2B in II). Previously we revealed that the FGFR4 variants regulate MT1-MMP activities at posttranscriptional levels (I). This may explain the low correlation of *FGFR4* and *MT1-MMP* mRNA expression in many types of carcinomas. However, there was one interesting exception; a positive correlation in prostate cancer. Therefore, in prostate carcinomas there is a possibility of FGFR4-MT1-MMP interplay that may contribute to cancer progression.

#### **4 FGFR4 regulates MT1-MMP-dependent ECM degradation and tumour progression in 3D collagen matrix and *in vivo* (II)**

Since the expression of endogenous FGFR4 and MT1-MMP are positively correlated in individual prostate carcinomas, we used prostate carcinoma cell lines to study how the FGFR4-MT1-MMP axis regulates tumour growth and invasion in 3D collagen matrices and mouse xenograft model. PC3 and DU145 human prostate carcinoma cell lines express both MT1-MMP and homozygous FGFR4-R388 risk variant or the FGFR4-G388 low risk variant, respectively (Sahadevan et al., 2007). The expression of FGFR4 and MT1-MMP was stably silenced by lentiviral shRNAs.

##### **4.1 FGFR4-R388 and MT1-MMP induce epithelial-to-mesenchymal transition (EMT) (II)**

In addition to invasion, MT1-MMP proteolytic activity enhances invasive cell growth within collagen matrices (Hotary et al., 2003). PC3 (R/R) and DU145 (G/G) cells after MT1-MMP or FGFR4 knockdown were implanted within three-dimensional cross-linked collagen I matrices as single cell suspension. The cells were allowed to grow for 6 days. Both PC3 and DU145 cells formed sphere-shaped colonies (Fig. 4A in II). Consistent with invasion abilities from atop collagen (Fig. 3 in II), PC3 colonies displayed more invasive protrusions and

invading individual cells, which were abolished by either the FGFR4-R388 or MT1-MMP knockdown (Fig. 4A in II). Consistent to our previous results with breast carcinoma cells (I), the FGFR4-R388 variant depletion in PC3 prostate carcinoma cells also decreased MT1-MMP protein levels and further increased the epithelial organization in the PC3 cells observed by increased cortical actin structure (Fig. 4A-C in II). In parallel, either the FGFR4-R388 or MT1-MMP knockdown increased E-cadherin levels coupled with suppression of cadherin-11 and N-cadherin expression (Fig. 4D in II). The FGFR4-R388 knockdown also reduced pro-migratory FRS2 and Src signalling, but not mitogenic signalling and cell proliferation in 2D culture (Fig. 4D and S5B-D in II). Since the expression balance between E-cadherin and N-cadherin or cadherin-11 is linked to EMT and the expression of N-cadherin and cadherin-11 can promote prostate cancer metastasis (Chu et al., 2008), the FGFR4-R388 variant expression can be involved in the induction of EMT and aggressive prostate cancer progression.

On the other hand, the FGFR4-G388 variant knockdown in DU145 cells up-regulated MT1-MMP protein levels and increased collective invasion within 3D collagen matrix (Fig. 4A, B, and S5A in II). MT1-MMP knockdown in turn increased the *FGFR4-G388* transcription (Fig. 4B and C in II). This suggests that there is a negative reciprocal regulation between MT1-MMP and the FGFR4-G388 variant in cancer cell invasion. FGFR4-G388 downregulates MT1-MMP protein levels and MT1-MMP knockdown increases its negative regulator, *FGFR4-G388* level. This FGFR4-G388-MT1-MMP axis can thus suppress DU145 cell invasion within 3D collagen matrices. The expression of the FGFR4-G388 variant did not affect cadherin expression, pro-migratory signalling, or cell growth (Fig. 4D and S5B-D in II).

#### **4.2 Endogenous FGFR4/MT1-MMP axis controls prostate carcinoma cell differentiation, extracellular matrix degradation, and EMT *in vivo* (II)**

To investigate the effects of the FGFR4-MT1-MMP axis on tumourigenesis *in vivo*, tumour growth, invasion, and collagen content were analysed after subcutaneously injection of PC3 and DU145 cells into SCID mice. The xenografts were allowed to grow for 6-8 weeks. The growth rates of PC3 tumours were dramatically decreased after stable knockdown of either MT1-MMP or the FGFR4-R388 risk variant (Fig. 5A in II). Mitotic index was decreased accordingly and PC3 cell invasion into vascular and lymph node capillaries was also impaired, while tumour collagen content was increased in these tumours (Fig. 5B and C, S6B, S7, and Table S3 in II). Consistent with *in vitro* study, FGFR4-R388 knockdown decreased MT1-MMP protein levels in mouse tumours as compared to control tumours (Fig. 6A in II). Since MT1-MMP is a major collagenase *in vivo* and its ECM remodelling activities promote cancer cell growth and invasion (Holmbeck et al., 1999; Hotary et al., 2003; Rowe and Weiss, 2008; Sabeh et al., 2009), the reduced MT1-MMP levels correlated with increased accumulation of collagen matrix within and around tumours as well as with impaired tumour growth and vascular and lymph node invasion in both MT1-MMP and the FGFR4-R388 variant depleted PC3 tumours. In contrast, the FGFR4-G388 low risk variant knockdown resulted in increased DU145 tumour growth and vascular invasion (Fig. 5 in II). This could be occurred by MT1-MMP upregulation, since the FGFR4-G388 variant depletion increased MT1-MMP protein levels *in vitro* and the increased MT1-MMP protein content was detected in particularly at the tumour edges (Fig 5 and 6A in II). Consistently, intratumoural collagen

and fibrous capsule around the tumours were decreased in the FGFR4-G388 depleted DU145 tumours (Fig. 5C in II). Thus, the FGFR4-G388 knockdown triggers MT1-MMP induction at the tumour edges, which could promote invasion of cancer cells that carry this low risk variant *in vivo* and within 3D collagen matrix.

Of note, the impaired growth and invasion of MT1-MMP and the FGFR4-R388 knockdown tumours were coupled with increased epithelial polarization and some acinar lumen formation (Fig. 6B in II). Furthermore, the basement membrane component, collagen IV was accumulated around the tumour cells, and cadherins were translocated to cell-cell junctions in these knockdown tumours (Fig. 6B in II). In contrast, the FGFR4-G388 silencing did not result in collagen IV accumulation in the DU145 tumours (Fig. S8D in II). Since mRNA levels of collagens (collagen IA, IIA, and IV) were not altered by MT1-MMP or FGFR4-R388 expression levels, the FGFR4-R388-MT1-MMP axis enhances the degradation of both basement membrane and fibrillar collagen components in the invasive PC3 tumours. Increased MT1-MMP on the cell surface can directly contribute to the induction of EMT by cleaving E-cadherin and other cell surface adaptor proteins (Ghajar and Bissell, 2008). Taken together, we demonstrated that the FGFR4 polymorphism can act as an activity switch of the FGFR4 and MT1-MMP complex *in vitro* and *in vivo*. At the stroma/tumour border or in the tumour invasive front the FGFR4-R388-MT1-MMP axis increases MT1-MMP proteolytic activities and FGFR4-FRS2-*Src* pro-migratory signalling, resulting in induction of EMT and promoting prostate tumour growth and invasion into adjacent interstitial tissues as well as into vessels by basement membrane and interstitial matrix degradation. The FGFR4-G388 low risk variant instead suppresses MT1-MMP levels at posttranscriptional levels and thus decreases prostate and breast carcinoma cell invasion. MT1-MMP in turn down-regulates this variant transcriptionally.

## **5 EphA2 cleavage by MT1-MMP triggers single cancer cell invasion via homotypic cell repulsion (III)**

EphA2 signalling regulates cell-cell and cell-matrix communications and cellular polarity and thus assists in maintaining normal tissue homeostasis (Miao and Wang, 2012). Aberrant expression or activation of EphA2 signalling can affect cancer cell-cell interactions and cancer cell motility through Rho-GTPase-mediated actomyosin contractility. EphA2 overexpression has been linked to aggressive progression of many types of cancer (Brantley-Sieders, 2012; Margaryan et al., 2009; Wykosky and Debinski, 2008). In glioblastoma and breast carcinomas, EphA2 overexpression is often coupled with low ephrinA1 expression (Macrae et al., 2005; Wykosky et al., 2005). However, the underlying cell-surface interactions and molecular mechanisms of EphA2-mediated cancer cell invasion have remained unclear.

### **5.1 EphA2 and MT1-MMP are co-expressed in invasive breast carcinoma cells (III)**

From the kinome screen, we identified EphA2 as a positive regulator for pro-invasive MT1-MMP in HT-1080 human fibrosarcoma cells. To study the potential relevance of such



regulation in cancer cell invasion, we first assessed the expression of EphA2 and MT1-MMP in non-invasive (ZR-75-1, MCF-7, BT-474, T47D, and MDA-MB-453; Neve et al., 2006) and invasive (SUM159, Hs578T, BT-549, and MDA-MB-231; Neve et al., 2006) breast carcinoma cell lines. Among them, EphA2 and MT1-MMP were selectively co-expressed in invasive cell lines where the EphA2 cognate ligand, ephrinA1 expression was low (Fig. 1B, C and S1A in III). Accordingly, phosphorylation of EphA2 was relatively poor (Fig. 1B, C and S1A in III). In contrast, in non-invasive breast carcinoma cells the expression of EphA2 and MT1-MMP was low coupled with expression of ephrinA1 (Fig. 1B, C and S1A in III; Neve et al., 2006).

Since EphA2 and MT1-MMP are up-regulated in invasive breast carcinoma cells, the effects of EphA2 on cell invasion were tested by invasion and invasive growth assays in cross-linked 3D collagen I matrix. Consistent with the expression of MT1-MMP, SUM159, Hs578T, BT-549, and MDA-MB-231 cells invaded into collagen matrices from atop collagen, which was impaired by either EphA2 or MT1-MMP knockdown by lentiviral shRNAs (Fig. 1E-G in III). These cells also grew efficiently within 3D collagen matrix and displayed variety types of invasive colony phenotypes (Fig. 2A in III). SUM159 and Hs578T cells that invaded more efficiently with elongated morphologies than MDA-MB-231 and BT-549 cells formed multicellular outgrowths rich in actin stress fibers (Fig. 1E, 2A-C and S1H in III). On the other hand, BT-549 cells grew in sphere-like colonies surrounded by rounded singly-invading cells with cortical actin. MDA-MB-231 cells had cortical actin coupled with more elongated morphology (Fig. 2A-C and S1H in III). EphA2 or MT1-MMP knockdown also reduced the invasive growth of these cells (Fig. 2D in III). Importantly, EphA2 depletion decreased *MT1-MMP* transcription and protein levels (Fig. 1H and S1C in III). Furthermore, EphA2 and MT1-MMP knockdown did not affect cell growth in 2D culture (Fig. S1I and J in III), thus suggesting that EphA2 may cooperate with MT1-MMP to regulate different types of cancer cell invasion within collagen matrices. MT1-MMP activity is likely to be regulated by EphA2 at transcriptional level.

One of the EphA2 downstream signalling, Src activation was impaired by EphA2 knockdown in BT-549 and MDA-MB-231 cells (Fig. 1I in III). Furthermore, EphA2 knockdown also decreased activation of Src in BT-549 and MDA-MB-231 cells (Fig. 1I in III). Src can regulate overall cell motility (Huveneers and Danen, 2009), and be associated with MT1-MMP activities (Barbolina et al., 2007; Nyalendo et al., 2007; Moss et al., 2009). Thus, Src activity can be involved in the potential cooperational regulation between EphA2 and MT1-MMP in breast carcinoma cell invasion.

## **5.2 MT1-MMP cleaves EphA2 to modulate receptor localization and cell junctional properties (III)**

To test the potential interaction of EphA2 and MT1-MMP in the different invasive breast carcinoma cells, we assessed the subcellular localization of these proteins in 2D culture. On plastic surface, SUM159 and Hs578T cells displayed mesenchymal-type phenotype with prominent actin stress fibres, while BT-549 and MDA-MB-231 cells displayed more cortical actin and inter-cellular spaces (Fig. 3B in III). We also observed the dispersed localization of EphA2 in SUM159 and Hs578T cells, whereas EphA2 was distinctly distributed to

perinuclear compartments and cell surface in BT-549 and MDA-MB-231 cells (Fig. 2A, 3B and S1K in III). In MDA-MB-231 cells, EphA2 knockdown increased actin stress fibres (Fig. 3C-E in III). MT1-MMP knockdown in turn reduced EphA2 accumulation in intracellular vesicles resulting in more dispersed localization and reduced inter-cellular spaces (Fig. 3C-E in III). Of note, either EphA2 or MT1-MMP knockdown did not alter EphA2 localization in SUM159 cells (Fig. S1J in III), although MT1-MMP was co-precipitated with EphA2 in SUM159, BT-549, and MDA-MB-231 cells (Fig. 3G in III). Similarly, Src inhibition stabilized EphA2 on the cell surface and dramatically reduced intracellular localization and cell-cell spaces (Fig. 3F in III). Therefore, MT1-MMP is associated with EphA2 intracellular localization and cell junctional properties through Src activation in some types of cells, such as MDA-MB-231 cells and BT-549 cells.

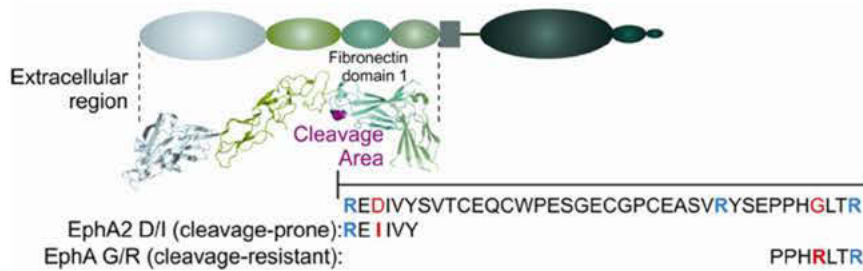
Interestingly EphA2 was prominently processed only in BT-549 and MDA-MB-231 cells, resulting in the formation of ~60 and ~50 kD fragments (Fig. 3H in III). The appearance of these fragments was reduced after MT1-MMP knockdown in MDA-MB-231 cells (Fig. 3I in III). Thus, MT1-MMP constitutively cleaves EphA2 and generates the ~60 kD and the ~50 kD fragments in these cells. The recombinant ephrinA1 treatment also increased ~50 kD fragment, which was a major fragment detected in BT-549 cells (Fig. 3H and I in III). The tested invasive breast carcinoma cells displayed different invasive phenotypes and EphA2 subcellular localization in 2D culture and within 3D collagen matrices. The physical EphA2-MT1-MMP interaction and constitutive cleavage of EphA2 was found in cortical actin-rich less elongated MDA-MB-231 cells and rounded BT-549 cells. In these cells EphA2 expression can also induce Src activation. Since low levels of ephrinA1 and other ligands are expressed in MDA-MB-231 and BT-549 cells (Macrae et al., 2005), upon cell-cell contact the expressing ligands in these cells can be sufficient to trigger limited receptor clustering and phosphorylation and further EphA2-dependent Src signalling activation. MT1-MMP may interact with EphA2 and cleave the receptor in the EphA2 clusters, leading to Src activity-dependent intracellular translocation of EphA2 and cell junctional disassembly. BT-549 cells express ephrin ligands at higher levels as compared to MDA-MB-231 cells (Macrae et al., 2005), which may reflect higher levels of ~50 kD fragment and more rounded single-cell phenotype of BT-549 cells within 3D collagen matrix. The cleavage of EphA2 was negligible in the mesenchymal-type SUM159 and Hs578T cells, even though MT1-MMP was co-precipitated with EphA2 in SUM159 cells. Therefore, the different types or magnitudes of receptor activation may be implicated in EphA2-MT1-MMP axis.

### **5.3 MT1-MMP cleaves EphA2 at fibronectin type-III domain *in cis* on the cell surface (III)**

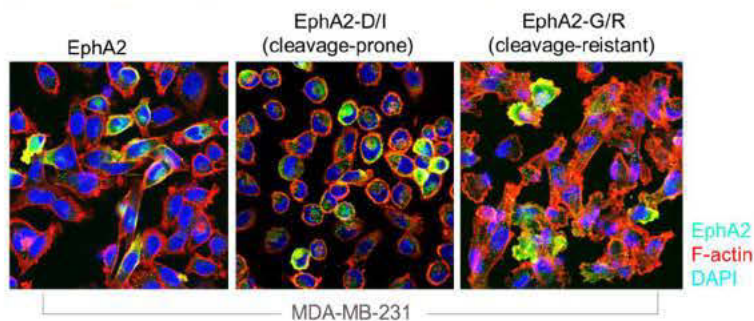
To identify the cleavage sites of EphA2 by MT1-MMP, we performed mass-spectrometry (LC-MS/MS) analysis. The cleavage area was located in Fibronectin type-III domain 1 (FN1; Fig. 4D and E in III). MEROPS database analysis (<http://merops.sanger.ac.uk/index.shtml>) revealed that the identified cleavage area contained the consensus and plausible substrate sequences for MT1-MMP. Further analyses showed that EphA2 was processed by MT1-MMP on the cell surface, when both proteins were expressed in the same cells (Fig. 4I and K in III). Previously it has been reported that upon binding of EphA receptor at cell-cell contacts, ephrinA was cleaved by adjacent cell surface ADAM proteases (Hattori et al., 2000;

Janes et al., 2005; Janes et al., 2009). This *in trans* ephrinA cleavage mediates repulsive cell-cell signalling (Hattori et al., 2000; Janes et al., 2005; Janes et al., 2009). Instead of ADAM-mediated ephrinA/EphA signalling at cell-cell junctions, the identified EphA2 cleavage by MT1-MMP is occurred on the same cell surface. Since MT1-MMP knockdown reduced inter-cellular spaces in MDA-MB-231 cell culture, this novel EphA2-MT1-MMP axis may also contribute to cell junction disassembly.

### 5.4 MT1-MMP-dependent EphA2 processing triggers cell-cell repulsion (III)



To test whether the EphA2 cleavage functionally contributes to the observed cell junction disassembly, we constructed cleavage-prone and cleavage-resistant EphA2 mutants based on MEROPS database analysis. While EphA2-D359I mutant increased the processing, EphA2-G391R previously found in human lung cancer cells conferred EphA2 with resistance to the cleavage (Figure 18; Fig. 4G and H in III; Faoro et al., 2010). Interestingly, the cleavage-prone EphA2-D/I was accumulated in the intracellular compartments and this mutant



expressing cells displayed rounded morphology and wider inter-cellular spaces (Figure 20). On the other hand, the cleavage-resistant EphA2 G/R expressing cells showed dispersed intracellular EphA2 localization concomitant with prominent stress fibre formation (Figure 21).

To study the possible contribution of EphA2 cleavage in cell junctional disassembly, we first assessed the effects of EphA2 and MT1-MMP on MDA-MB-231 cell movement in 2D culture by live cell imaging. Control cells moved freely long distances and underwent numerous steps of adhesion and detachment (Fig. 5A and Movie S2 in III). EphA2 knockdown reduced detachments and overall cell movement, whereas MT1-MMP depletion specifically hindered the detachment of colliding cells (Fig. 5A and Movie S3 and S4 in III). We next assessed whether EphA2 cleavage affects colliding cell detachment using wild-type EphA2 and cleavage-prone EphA2-D/I expressing MDA-MB-231 cells. Overexpression of EphA2 did not affect cell-cell interactions. However, the EphA2-D/I strongly decreased the number of non-detaching cells, which was cancelled by MT1-MMP knockdown (Fig. 5C; Movie S5-10 in III). This cleavage-prone mutant further triggered significantly greater

directional switches upon collision, depending on MT1-MMP activity (Fig. 5D-F, S2D and E in III). Therefore, the EphA2 cleavage by MT1-MMP can promote contact-mediated repulsive cell movement upon EphA2 cleavage.

### **5.5 Prominent EphA2 cleavages promote RhoA activation and single-cell invasion within 3D collagen matrices (III)**

Since the higher levels of constitutive EphA2 cleavage in BT-549 cells is appeared to be correlated with more single-cell invasion phenotype within 3D collagen matrices, we assessed if the cell repulsion triggered by EphA2 cleavage is linked to different types of cancer cell invasion. MDA-MB-231 cells expressing EphA2, EphA2-D/I, EphA2-G/R, and the kinase activity-deficient proteins (EphA2-KD and EphA2-KD-D/I) were implanted in cross-linked 3D collagen matrices as single cell suspension. Control and EphA2 expressing cells formed coherent cell colonies, where EphA2 was detected in the cell surface and intracellular compartments (Fig. 6A in III). The cleavage-prone EphA2-D/I expression resulted in single-cell invasion within 3D collagen, while the cleavage-resistant EphA2-G/R cells formed large collectively invading colonies (Fig. 6A-D in III). Consistent with 2D culture, EphA2-D/I was mainly detected in the intracellular vesicles and EphA2-G/R was dispersed in the cytoplasm (Fig. 6A in III). Increased EphA2 cleavage and intracellular localization increased RhoA activation and phosphorylation of myosin light chain (MLC) in both 2D and 3D culture (Fig. 6E and S3C in III). When the EphA2 kinase activity was impaired, the cells expressing EphA2-KD or EphA2-KD-D/I protein formed coherent colonies with multicellular sprouts with mesenchymal-type elongated cells, even though the EphA2-KD-D/I was efficiently cleaved in ~60 kD fragment (Fig. 6A-D and S3A in III). These KD proteins appeared to decrease the activation levels of RhoA (Fig. 6E in III), thus catalytically active EphA2 kinase activity is required for breast carcinoma cell transition to more rounded morphology. Since ligand-induced EphA2 signalling leads to cell rounding coupled with loosening cell-cell and cell-matrix interactions (Miao et al., 2000; Parri et al., 2009; Taddei et al., 2011), upon cell collision EphA2-MT1-MMP-Src signalling can trigger cell body retraction and cell junctional dissociation via RhoA-mediated actomyosin contraction (Fig. 10 in III). Increased *MT1-MMP* expression by EphA2 may further promote effective EphA2-D/I expressing single-cell invasion within collagen. In 3D condition, cell junctional assembly, integrin-mediated cell-ECM adhesion, pericellular ECM degradation can differently regulate cell motility and cell phenotype from 2D condition (Friedl and Alexander, 2011; Harunaga and Yamada, 2011; Sabeh et al., 2009). However, the MT1-MMP-EphA2 axis regulates RhoA-mediated cell repulsive movement in both 2D and 3D cultures, thus suggesting that EphA2 cleavage and EphA2-MT1-MMP interaction can more uniformly function in cancer cell invasion on sheet-like BMs as well as within 3D interstitial tissues. Furthermore, EphA2 is cooperated with other RTK signalling to promote collective cell invasion in ligand-independent manner (Hiramoto-Yamaki et al., 2010; Miao and Wang, 2009). The potential cross-talk between EphA2 and other RTK signalling can be thus one mechanism of mesenchymal-type cell invasion.

## 5.6 EphA2 cleavage promotes single cell dissemination *in vivo* (III)

To study if the EphA2-MT1-MMP axis regulates cancer invasion phenotype *in vivo*, control and EphA2 or EphA2-D/I stably-expressing MDA-MB-231 cells were orthotopically injected into the mammary fat pad of SCID female mice. All xenografts formed tumours within 4 weeks. The difference in the growth rate of each xenograft was minor, however each tumour showed different cancer cell phenotype. Control and EphA2 expressing cells grew cohesively, while the cells in EphA2-D/I tumours dissociated cell-cell junctions and invaded as single cells into stroma (Fig. 8A, B, E and S3G in III). In the metastatic lymph nodes, the control cells remained mainly within cortex with less MT1-MMP expression compared to the primary tumours (Fig. 8C, D, S3E and F; metastasis in sentinel lymph nodes; 2/5 in III). Since cancer cells switch their phenotypes from motile invasive to growth phenotype in the metastatic sites (Giampieri et al., 2009), the decreased MT1-MMP level in the lymph nodes of control tumour-bearing mice can be a sign of this transition. EphA2 expressing cells formed coherent metastatic colonies, the metastatic EphA2-D/I instead displayed poor cell-cell contacts even more than in primary tumours (Fig. 8C-E and S3E-G; metastasis in sentinel lymph nodes; 2/5; metastasis in sentinel lymph nodes of EphA2-D/I expressing cells; 4/5 in III). Consistent with EphA2 effect on *MT1-MMP* transcript (Fig. 1H and S1C in III), EphA2 increased MT1-MMP throughout the tumours (Fig. 8 and S3E-G in III). Importantly, the cleavage-prone EphA2-D/I increased MT1-MMP at higher levels as compared to EphA2 *in vivo* (Fig. 8 and S3E-G in III). Although the mechanism remains to be elucidated, cleaved intracellular active EphA2-inducible signalling may directly or indirectly increase MT1-MMP levels, facilitating cancer invasion and cell phenotype switch from collective to single-cell mode *in vivo*.

To further study the relevance of the EphA2-MT1-MMP axis in human breast carcinoma progression, expression and localization of EphA2 and MT1-MMP were assessed by immunohistochemistry using human tumour array containing invasive breast tumours with matched normal tumour-adjacent tissue or lymph node metastasis. EphA2 is typically expressed in tumour epithelium, while MT1-MMP expression is induced at invasive edge and reactive stroma (Brantley-Sieders et al., 2011; Okada et al., 1995; Sabeh et al., 2009; Sugiyama et al., 2010; Szabova et al., 2005). Consistently, EphA2 was detected in luminal epithelium and carcinoma cells and MT1-MMP was in both the carcinomas and reactive stroma (Fig. 9A, B and S4 in III). Expression of both EphA2 and MT1-MMP was high in invasive breast carcinomas as compared to tumour-adjacent normal tissues. Interestingly, the intracellular EphA2 co-localized with the up-regulated MT1-MMP in invasive edge and dissemination cancer cells within stroma of human breast carcinomas (Fig. 9A, B and S4 in III). Therefore, the EphA2 cleavage and EphA2-MT1-MMP interaction can function in context-dependent cancer cell-cell communications *in vivo*.

## CONCLUSIONS AND PERSPECTIVES

Cancer invasion promotes cancer cell dissemination from a locally growing tumour to regions throughout the body and thus becomes a major clinical problem. In this thesis we aimed to identify novel protein kinases that regulate pro-invasive MT1-MMP activities in cancer cells. The FGFR4-R388 variant and EphA2 were identified as positive regulators that cooperate with MT1-MMP to regulate cancer progression and cancer invasion plasticity.

FGFR4-R388 is a germline SNP variant of FGFR4. Approximately half of Caucasian and Asian human population are bearing the FGFR4-R388 allele (Bange et al., 2002; Seitzer et al., 2010; Stadler et al., 2006; Wang et al., 2004). This G388R SNP in FGFR4 positively correlates with prostate cancer risk and aggressive progression of multiple types of cancer. We demonstrate that upon interaction of the FGFR4 variants and MT1-MMP, the SNP functions as a switch of MT1-MMP activity in cancer. The FGFR4-G388 low risk variant and MT1-MMP suppress each other's expression. In contrast, FGFR4-R388 risk variant and MT1-MMP enhance pro-migratory FGFR4-FRS2-Src signalling and MT1-MMP proteolytic activities, promoting EMT and prostate cancer invasion and invasive growth in mouse xenograft models. Due to the opposite effects of FGFR4 variants on MT1-MMP activity, unselective targeting of FGFR4 would be expected to result in either beneficial or harmful outcome in cancer patients. In our experimental model, FGFR4-G388 gene silencing induced upregulation of MT1-MMP and increased tumour spread. We thus anticipate that FGFR4 SNP genetic background screening and selective targeting to the FGFR4-R388 variant by generating specific monoclonal antibodies or small molecules would be useful for personalized cancer therapy.

The EphA2-MT1-MMP axis affects breast carcinoma cell invasion modes between collective and single-cell phenotype that can contribute to cancer cell metastasis and escape responses to cancer therapy. As summarized in Fig. 10 in III, at cell-cell junctions or upon cell collision few ephrin ligands can induce EphA2 clustering and activation. The cleavage of active EphA2 by MT1-MMP in protein complexes on the same cell membrane surface triggers the receptor internalization by EphA2-dependent Src activity, leading to cell body contraction through RhoA activation. This EphA2-MT1-MMP-Src-RhoA signalling regulates repulsive cell movement and cell junctional disassembly in both 2D and 3D environment, and ultimately single-cell dissemination within 3D collagen matrix and *in vivo*. Importantly, activation and cleavage levels of EphA2 and EphA2-MT1-MMP interaction can regulate different types of cancer cell invasion phenotype. For examples, co-expression of MT1-MMP and cleavage-prone EphA2-D359I mutant enhances contact-mediated cell repulsion and ECM-degradation, resulting in effective rounded single-cell invasion. In contrast, the cleavage-resistant EphA2-G391R mutation found in lung cancer induces rapidly and collectively invasive cell colony growth, while the kinase activity-deficient proteins expressing cells formed cohesive cell colonies with mesenchymal-type elongated cell protrusions. Thus, the EphA2-MT1-MMP axis can function in the distinct stage of tumour progression, e.g. tumour invasive growth, collective invasion, and single-cell invasion. Understanding the regulatory mechanism of EphA2 cleavage and signalling in distinct tumour contexts could be helpful in developing strategies for efficient clinical interventions for cancer patients in future. Furthermore, generation of monoclonal antibodies or small

molecules that selectively bind to the cleavage area of EphA2 would be predicted to inhibit EphA2 cleavage and intracellular compartmentalization, which may help to palliate metastatic cancer progression by preventing up-regulation of MT1-MMP and cancer cell invasion plasticity.

The broad spectrum synthetic MMP inhibitors have had limited clinical success due to low selectivity of inhibitors and because of multiple functions of MMP family members (Devy and Dransfield, 2011; Zucker and Cao, 2009). The findings of this thesis study would help to alleviate the cancer promoting activities of MT1-MMP by revealing how FGFR4 and EphA2 co-operate with MT1-MMP during cancer progression. This study could motivate to develop strategies of more effective and less toxic anti-invasion and anti-metastasis cancer therapies.

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