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FROM EPITHELIAL TO MESENCHYMAL: REGULATION OF INVASIVE CANCER CELL MOTILITY

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To my family

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From epithelial to mesenchymal: regulation of invasive cancer cell motility

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ABSTRACT

Metastasis is the main cause of death among cancer patients. In order to initiate the metastatic cascade cancer cells have to undergo epithelial-to-mesenchymal transition (EMT). In EMT epithelial cells lose their cell-cell and cell-extracellular matrix (ECM) contacts and become more motile. The expression of the transcription factor Slug and of the mesenchymal intermediate filament vimentin is induced during EMT. Vimentin is often overexpressed in malignant epithelial cancers but the functional role of vimentin remains incompletely understood. In addition, kinases such as AKT and ERK are known to be involved in the regulation of EMT and cancer cell motility but the mechanisms underlining their functions are often unclear. Integrins are heterodimeric receptors that attach cells to the surrounding tissue and participate in regulating cell migration and invasion. Changes in integrin activity are linked to increased cell motility and further cancer metastasis.

The aim for my PhD studies was to investigate the role of cellular signalling pathways and vimentin in the regulation of cancer cell motility and EMT. Our results revealed that in prostate cancer the downregulation of AKT1 and AKT2, but not AKT3, induces activation of cell surface β1-integrins leading to enhanced cell adhesion, migration and invasion. In addition, our findings demonstrated a reciprocal regulatory interaction between vimentin and ERK2 facilitating ERK-mediated phosphorylation of Slug at serine-87 (S87) in breast cancer. Surprisingly, Slug S87 phosphorylation is dispensable for E-cadherin repression but essential for the induction of vimentin and Axl expression in early onset of EMT. Our findings reveal previously unknown mechanistic information of how prostate and breast cancer cell motility and disease progression is regulated.

Keywords: Integrin activity, signalling, vimentin, transcription factor

Reetta Virtakoivu
Epiteelisestä mesenkymaaliseksi: invasoivien syöpäsolujen liikkumisen säätely

Turun yliopisto, Biolääketieteen laitos, Lääketieteellisen Biokemian ja genetiikan oppiaine, Turun Biotekniikan keskus, VTT Lääkekehityksen biotekniikka, Molekyylilääketieteen tohtoriohjelma ja Turun Biolääketieteellinen tutkijakoulu, Turku, Suomi
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TIIVISTELMÄ

Syövän leviäminen eli metastasointi ja etäpesäkkeiden muodostuminen on syöpäpotilaiden yleisin kuolinsyy. Jotta monivaiheinen metastasointi prosessi voi alkaa, syöpäsolujen on käytävä läpi epiteeli-mesenkyymimuutos (EMT). EMT prosessin aikana epiteelisolut menettävät solujen väliset kontaktinsa ja muuttuvat pahanlaatuisiksi mesenkyymisoluiksi. Transkriptiotekijä Slugin ja välikokoisten säikeiden proteiiniperheeseen kuuluvan vimentiinin ilmentyminen lisääntyy EMT prosessissa. Tämän lisäksi vimentiinin ilmentymisen tiedetään lisääntyneen pahanlaatuisissa epiteelisyövissä, mutta tämän merkitystä tai vimentiinin toiminnallista roolia ei tiedetä. AKT ja ERK kinaasien on näytetty osallistuvan EMT prosessin kannalta tärkeiden proteiinien säätelyyn, mutta yksityiskohtaisista säätelymekanismeista on vain vähän tietoa. Integriinit ovat solunpinnan tarttumisreseptoreita, jotka välittävät solujen kiinnittymistä toisiin soluihin tai solun ulkoiseen väliaineeseen. Niiden aktivoitumisen tiedetään olevan tärkeää solujen liikkumiselle ja siksi integriinien aktivoitumisen uskotaan osallistuvan syövän leviämiseen.

Väitöskirjatutkimukseni tavoitteena oli tutkia solujen signaloinnin ja solun tukirangan säieproteiini vimentiinin merkitystä syöpäsolujen liikkumisessa ja etäpesäkkeiden muodostuksessa. Tulokseni näyttävät eri AKT isomuotojen säätelevän eturauhassyöpäsolujen liikkumista eri tavoin. Saadut tulokset osoittavat sekä AKT1 että AKT2 kinaasin toiminnan estämisen lisäävän β1-integriini aktiivisuutta ja edelleen johtavan eturauhassyöpäsolujen lisääntyneeseen migraatioon ja invaasioon. Sen sijaan AKT3 kinaasin toiminnan estäminen ei vaikuta näihin toimintoihin. Väitöskirjassani tutkittiin myös EMT prosessin säätelyä rintasyövässä. Saadut tulokset osoittavat, että vimentiinin ja ERK2 kinaasin välinen vuorovaikutus johtaa Slug transkriptiotekijän fosforylaatioon ERK spesifisesti. Tämä Slug seriini 87 kohdan fosforylaatio on tärkeää Slugin kyvylle indusoida vimentiinin ja Axl reseptorin ilmentymistä EMT prosessin alkuvaiheessa. Löydösten avulla saatiin uutta tietoa mekanismeista, jotka säätelevät eturauhas- ja rintasyöpäsolujen liikkumista ja metastasointia.

Avainsanat: Integriinien aktiivisuus, signalointi, vimentiini, transkriptiotekijä

TABLE OF CONTENTS

ABBREVIATIONS

ADT	Androgen deprivation therapy
AP	Alkaline phosphatase
AR	Androgen receptor
bFGF	Basic fibroblast growth factor
BM	Basement membrane
BRCA 1/2	Breast cancer 1/2
CDK1	Cyclin-dependent kinase 1
CRPC	Castration-resistant prostate cancer
CTC	Circulating tumour cell
DTC	Disseminated tumour cells
DUSP	Dual-specificity phosphatase
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
ES	Embryonic stem cell
ESRP	Epithelial splicing regulatory protein
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FRA1	FOS-related antigen 1
FRAP	Fluorescence recovery after photobleaching
GF	Growth factor
GFAP	Glial fibrillary acidic protein
GFR	Growth factor receptor
GPCR	G-protein-coupled receptors
GSK3β	Glycogen synthase kinase 3β
HGFR	Hepatocyte growth factor receptor
HER	Human epidermal growth factor receptor
HSP90	Heat shock protein 90
IF	Intermediate filament
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IKK	IκB kinase
ILK	Integrin-linked kinase
INPP4B	Inositol polyphosphate 4-phosphatase B
IP	Immunoprecipitation
kD	Kilodalton
ko	Knock-out
LRHR	Luteinizing-hormone-releasing hormone

MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2 homolog
MEF	Mouse embryonic fibroblast
MET	Mesenchymal-to-epithelial transition
MKP	MAP kinase phosphatase
MMP	Matrix metalloproteinase
PAK	p21-activated kinase
PDGF	Platelet-derived growth factor
PH	Pleckstrin homology
PIP3	Phosphatidylinositol (3,4,5)-triphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLK1	Polo-like kinase
PP2A	Protein phosphatase 2A
PTEN	Phosphatase and tensin homolog
PTB	Phosphotyrosine-binding domain
qRT-PCR	Quantitative real-time polymerase chain reaction
Rluc	Renilla luciferase
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SERD	Selective estrogen receptor downregulator
SERM	Selective estrogen receptor modulator
siRNA	Small interfering RNA
STED	Stimulated emission depletion
STS	Soft tissue carcinoma
TGFβ	Transforming growth factor beta
TNBC	Triple-negative breast cancer
TNF-α	Tumour necrosis factor alpha
TSC	Tuberous sclerosis
TWIST	Twist-related protein
VEGF	Vascular endothelial growth factor
WB	Western blotting
wt	Wild-type
ZEB	Zinc finger E-box-binding homeobox
ZO-1	Zonula occludens protein-1

LIST OF ORIGINAL PUBLICATIONS

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

I **Distinct roles of AKT isoforms in regulating β1-integrin activity, migration and invasion in prostate cancer**
Virtakoivu R, Pellinen T, Rantala JK, Perälä M, Ivaska J.
Mol Biol Cell 2012

II **Vimentin-ERK signaling uncouples Slug gene-regulatory function**
Virtakoivu R, Mai A, Mattila E, De Franceschi N, Imanishi SY, Corthals G, Kaukonen R, Saari M, Cheng F, Torvaldson E, Kosma VM, Mannermaa A, Muharram G, Gilles C, Eriksson J, Soini Y, Lorens JB, Ivaska J.
Cancer Res. 2015

1. INTRODUCTION

Approximately 90 % of all human cancers originate from epithelial tissue. Cancer cells grow uncontrollably leading to the formation of malignant tumours, metastasis and death. The prevalence of cancer is increasing constantly and cancer has become the leading cause of death in the western world. Epithelial cancers called carcinomas become lethal after metastasis have occurred and this is a major challenge for combating cancer. For cancer cells to leave the primary tumour, migrate into the surrounding tissues and form metastases, they have to go through epithelial-to-mesenchymal transition (EMT). In EMT, epithelial cells detach their cell-cell contacts and adopt a more fibroblast-like mesenchymal phenotype. This process is closely linked to metastasis and cancer progression (Thiery and Sleeman 2006). The intermediate filament (IF) protein vimentin is normally expressed in cells of mesenchymal origin and it is considered to be a marker of EMT in cancer. The IF family of proteins are one of the three main components that form the cytoskeleton. IFs have been shown to participate in organizing proteins involved in cell signalling, adhesion and migration (Ivaska, Pallari et al. 2007). In addition to IFs several transcription factors, like Slug (SNAI2) and Snail (SNAI1), have been linked to cancer cell motility and are known to regulate EMT. The activity of these transcription factors is a result of complex regulation of protein stability, and subcellular localization, via different phosphorylation events (Lamouille, Xu et al. 2014). Kinases linked to EMT, such as protein kinase B (PKB/AKT) and mitogen-activated protein kinase (ERK2), are known to participate in this regulation (Irie, Pearline et al. 2005, Shin, Rath et al. 2010). In breast cancer, EMT characteristics are enriched in the aggressive and metastatic triple-negative breast cancer subtype (TNBC) (Shah, Roth et al. 2012), suggesting a role for EMT in breast cancer metastasis.

Integrins are transmembrane receptors that attach a cell to the surrounding tissue and participate in regulating cell proliferation, movement and survival. A prerequisite for cancer cells to start migrating is to lose their epithelial polarisation and cell-cell contacts as well as to alter their contacts with the extracellular matrix (ECM). Changes in integrin activity are linked to increased cell motility (Hynes 2002). In addition, several integrin heterodimers can be up- or downregulated in cancer (Desgrosellier and Cheresh 2010).

The aim for my PhD studies was to investigate the link between cancer cell motility and EMT and to delineate the molecular pathways regulating these processes. Particularly, I was interested in studying the relationship between vimentin, and key regulators of EMT, and integrin function. Understanding the mechanisms regulating EMT induction and cancer cell migration, would help to unravel the details behind cancer cell metastasis and potentially reveal new approaches for targeting EMT and metastasis.

2. REVIEW OF THE LITERATURE

2.1 CANCER METASTASIS

2.1.1 Cancer formation

Carcinogenesis is a long process where a normal cell has to undergo several changes to become a cancer cell. This multistep process involves genetic and epigenetic changes of multiple genes. Genetic changes like gene amplifications, mutations, chromosomal translocations and deletions often target proto-oncogenes and tumour suppressor genes. In cancer, the activation of proto-oncogenes, for example by gain-of-function mutations, leads to increased proliferative signals and uncontrolled tumour growth. In contrast, tumour suppressor genes are often inactivated for instance via DNA methylation, and are therefore no longer able to inhibit cancer cell growth. A small portion of carcinoma-inducing changes can be inherited like breast cancer 1/2 (BRCA1/2) mutations which increase the risk of developing breast or ovarian cancer up to 65 percent (the risk of developing breast and ovarian cancers among women in the normal population is 12 and 1.3 percent, respectively) (National Cancer institute). However, the majority of carcinomas result from somatic mutations triggered by external cues such as environmental carcinogens like chemicals, radiation and diet. In addition, aging is one of the biggest risk factors in developing cancer. Spontaneous mutations accumulate in aging, inducing genomic instability and sensitivity towards carcinogens and tumour promoters (Anisimov 2009, de Magalhaes 2013). Together, these factors can induce genetic alterations over time and once there are enough changes, a normal cell may transform into a cancer cell. There is now clear evidence showing the importance of the cell microenvironment for tumour development. Cross-talk between normal and malignant cells and interplay with the microenvironment influences tumour formation as well. Transformed malignant cells can send growth-inducing signals to neighbouring normal cells in the surrounding stroma and induce them to secrete growth factors (Barrett 1993, Barcellos-Hoff, Lyden et al. 2013).

According to Hanahan and Weinberg there are eight main characteristics and two supporting features that normal cells have to gain before evolving into a cancer cell (Hanahan and Weinberg 2011). These main characteristics are: sustained proliferation, the ability to avoid growth suppressors and to resist apoptosis, infinite replication capacity, formation of angiogenesis, induction of invasion and metastasis, re-programming energy-metabolism and the ability to overcome immune destruction. The supporting features are genomic instability and tumour-promoting inflammation. Uncontrollable proliferating cells are defined as cancer cells so it is not surprising that deregulation of the cell cycle is one of the hallmarks of cancer. Induction of growth factor receptor (GFR) signalling by activating mutations in receptors or by continuous production of

growth factors (GFs) leads to hyper proliferation of cancer cells. Tumour-suppressor genes are important in limiting cell growth and proliferation. They negatively regulate the cell cycle by repressing the expression of genes that are needed for cells to cycle, by stopping the cell cycle after DNA damage or by inducing apoptosis and contact inhibition. One of the well-known tumour-suppressor gene products is p53 which is frequently mutated and inactivated in different cancer types. In response to cellular stress including DNA damage or oncogene activation, p53 arrests growth by halting the cell cycle at G1/S checkpoint. P53 activates DNA repair proteins and if the damage cannot be fixed p53 signalling promotes apoptosis. The ability to resist apoptosis is a feature that cancer cells have obtained. Apoptosis is primarily triggered by extracellular death-inducing signals or by integration of internal apoptotic signals. This leads to activation of proteases, generally caspases 8 and 9. In addition, in healthy cells, telomere repeats at the end of telomeric DNA are shortened in every cell division. Cancer cells express the specialized DNA polymerase called telomerase that adds telomere repeats to the end of telomeric DNA, allowing cells to divide infinitely. Overall, there are several ways by which cancer cells can escape programmed cell death and promote uncontrollable proliferation leading to formation of tumours.

When the primary tumour size increases, the tumour requires nutrients and oxygen and the removal of waste products. To overcome these issues during tumour growth, new vasculature is formed in a process called angiogenesis. Activators of angiogenesis like vascular endothelial growth factors (VEGFs) are often upregulated in cancer. Local invasion and metastasis to distant organs involves alterations in cell shape and cell-cell contacts and these changes often occur during tumour progression. Reprogramming energy metabolism is one of the two emerging hallmarks of cancer. During this process cancer cells limit their energy metabolism to glycolysis to produce lactate even in the presence of oxygen in a process called “aerobic glycolysis”. The upregulated lactate promotes cancer cell migration and clustering (Hirschhaeuser, Sattler et al. 2011). The other emerging hallmark is the ability of cancer cells to overcome immune destruction by escaping the immune system. In addition to these hallmarks of cancer, Hanahan and Weinberg describe two enabling characteristics. These two capacities backup the cancer cells. Genomic instability exposes cancer cells to a great number of random mutations and tumour-promoting inflammation is a state where non-carcinogenic tissues mirror the inflammatory conditions.

2.1.2 Prostate cancer

Prostate cancer is the second most common cancer among men after lung cancer. More than 90% of prostate cancer incidents occur in men over 50 years of age and only a small proportion of these incidents are metastatic. The non-spread prostate cancer is treated with radiotherapy or by prostatectomy or

merely by “watchful waiting” meaning active monitoring of the tumour without any treatment. In addition to these treatments, advanced metastatic prostate cancer requires androgen deprivation therapy (ADT) but often remains incurable (Shen and Abate-Shen 2010). Androgen receptor (AR) is the main target of hormone therapy. At the stage when prostate cancer is diagnosed, it is dependent on AR signalling and as a result is sensitive to hormonal treatments such as the luteinizing-hormone-releasing hormone (LRHR) agonist. However, almost all patients develop the more aggressive castration-resistant prostate cancer (CRPC) within 18-24 months after initiation of treatment. Development of CRPC is the principal cause of death among prostate cancer patients (Sharma, Massie et al. 2013, Gundem, Van Loo et al. 2015).

2.1.3 Breast cancer

Breast cancer is the most common cancer among women worldwide. In recent years it has become evident that in breast cancer there is huge heterogeneity between tumours and even within the same tumour. This heterogeneity poses a major challenge in the treatment of breast cancer. There are different classifications of breast tumours but one commonly used classification divides the disease into five subtypes: luminal A, luminal B, human epidermal growth factor receptor (HER2)-positive, normal-like and basal-like breast cancer (Figure 1).

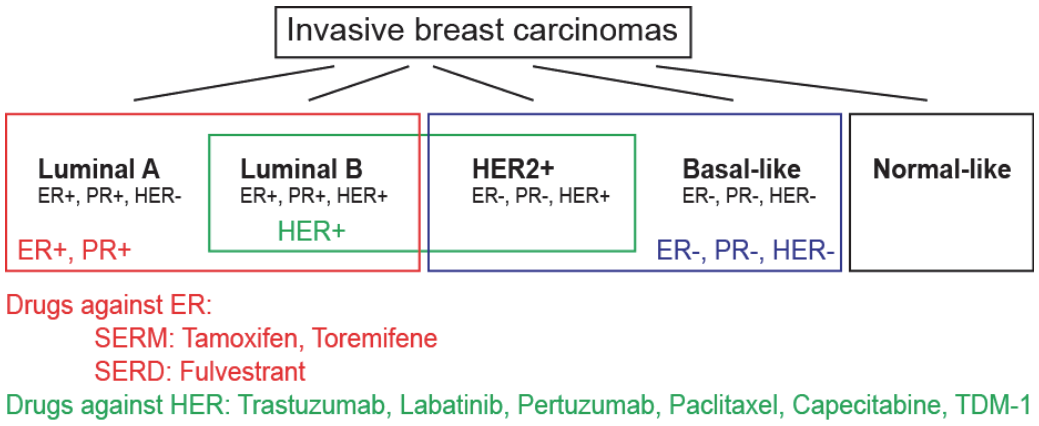

Figure 1. The different subtypes of breast cancer. Breast tumours are categorized into five different subtypes according to the hormone receptor status on the cell surface. The most common drugs used against these receptors for breast cancer treatment are shown. SERM, selective estrogen receptor modulator, SERD, selective estrogen receptor downregulator, ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor. + stands for positive; - for negative.

Treatment of breast cancer patients is determined based on this classification and the most common drug compounds are shown in figure 1. Many of these therapeutic agents target HER2 or ER. The basal-like subtype is also referred to as TNBC. Since TNBC is negative for estrogen, progesterone and HER2 receptors, there are fewer options to treat this subtype and this is why it has the poorest survival prognosis (Bartholomeusz, Gonzalez-Angulo et al. 2012, Liu, Zhang et al. 2014, Di Leo, Curigliano et al. 2015).

2.1.4 Cancer metastasis

More than 90 % of cancer-related deaths are due to metastasis. In many cancer patients at the time of diagnosis, micrometastases have already formed although these cannot be visualized with conventional methods (Talmadge and Fidler 2010). Metastasis is a complex process consisting of primary tumour progression, metastatic dissemination and colonization to distant organs. For example breast cancer cells form metastasis often to the lungs, bones and brain, whereas advanced prostate cancer predominantly metastasises to the bones. There are several obstacles that transformed cancer cells have to overcome before formation of secondary tumours in distant organs. So even though there are hundreds of cells migrating out from the primary tumour and circulating tumour cells (CTCs) are frequently detected in cancer patients, only a small subset of these cells will survive and manage to colonize in a distant organ (Jiang, Sanders et al. 2015).

The first step of the metastatic process is local invasion. The primary tumour is surrounded by a basement membrane (BM) which functions as a border between the tumour and the underlying stroma. When the tumour size exceeds 1 to 2 mm in diameter, simple diffusion of nutrients and oxygen is not enough to fulfil the energy requirements of the tumour. The reduction in oxygen levels (hypoxia) is often the driving force for initiation of angiogenesis and the metastatic cascade (Lu and Kang 2010). For tumour cells to invade the local surroundings the basement membrane has to be degraded. This breakdown of basement membrane resulting from proteolytic degradation of ECM components is thought to be a critical step for invasion and metastasis (Zeng, Cohen et al. 1999). Invading epithelial cancer cells themselves can penetrate the basement membrane by using actin-rich structures called invadopodia. Additionally, macrophages at the tumour periphery can positively induce basement membrane and matrix degradation by supplying matrix-degrading enzymes such as matrix metalloproteinases (MMPs). Tumour-associated fibroblasts have also been implicated in the degradation of the basement membrane (Kelley, Lohmer et al. 2014). When cancer cells have migrated and invaded the surrounding tissues, there are new obstacles to avoid such as immune surveillance; tumour-antagonizing cytotoxic T cells and natural killer cells. The strongest of cancer cells that manage to survive will proceed to the next step of the metastatic cascade called intravasation. During intravasation

the cancer cells infiltrate local blood and lymphatic vessels. To survive in the circulation cancer cells have to cope with multiple stresses like physical damage caused by hemodynamic shear forces and immune cell mediated killing. The surviving cancer cells are called circulating tumour cells (CTCs). To leave the circulation, CTCs have to attach to the endothelium and invade into a target tissue in a process called extravasation. Alternatively, cancer cells can disseminate following entrapment into the thin vessels of the microvasculature. Disseminated cancer cells preferably form metastases in distinct organs. What remains unknown is whether the cancer cells disseminating from the primary tumour already at that point are destined for specific organs (Gupta and Massague 2006, Coghlin and Murray 2010). Paget´s 126-year-old “seed and soil” hypothesis proposed that the distribution of metastases is not random. It rather depends on the interaction between disseminated cancer cells and specific organ microenvironments (Paget 1989).

The colonization of tumour cells, meaning the initiation of micrometastases and subsequent tumour growth, is the rate limiting and last step of metastasis. When cancer cells have reached the site of metastasis they have to adapt to the unfamiliar microenvironment and undergo cell proliferation to form micrometastases followed by macrometastases. In order to do so, disseminated cancer cells have to overcome several obstacles to promote tumour cell survival, like remodelling the ECM to form a tumour-supportive microenvironment and eluding immune surveillance. Only a very small proportion of these cells will manage to proliferate into fully malignant secondary tumours, whereas most will die or survive but remain unable to proliferate (dormancy) (Figure 2) (Shibue and Weinberg 2011).

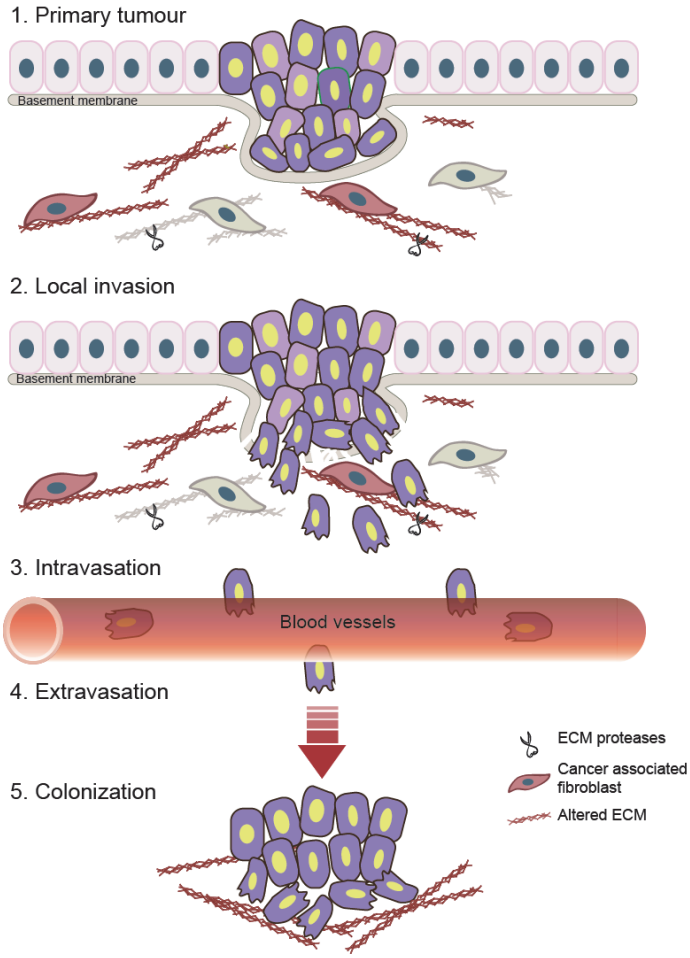

Figure 2. The metastatic cascade. To initiate the metastatic spread of cancer, epithelial tumour cells have to become more motile and degrade the underlying basement membrane and ECM. Local invasion is followed by intravasation during which the tumour cells penetrate into the blood or lymphatic vessels and enter the circulation. During extravasation the surviving tumour cells attach to, and invade through, the capillary endothelium into the parenchyma of distant tissues. The formation of micrometastases in an unfamiliar microenvironment and proliferation into malignant secondary tumours is called colonization.

2.1.5 Integrin receptors

Integrins are cell adhesion molecules that participate in cell-cell and cell-ECM interactions. They are transmembrane receptors consisting of an α and a β subunit. In humans there are 18 known α and 8 β subunits which can form 24 distinct heterodimers. Integrins can be found in three different conformational

states in cells; inactive (closed), extended (primed) or active (open) conformation, depending on their activation status. The activation of integrins can occur by outside-in or inside-out activation. In outside-in activation integrins bind to ECM components like fibronectin, collagen or laminins and this induces an integrin conformation shift from closed to extended state. This is followed by the binding of talin or kindlin, the best known activators of integrins, to β integrin cytoplasmic tails. This binding triggers the separation of the α- and β-tails which fully opens up the integrin conformation. Other adaptor and scaffolding proteins can bind to this fully activated integrin and finally couple the actin cytoskeleton to the ECM. In inside-out activation the stimulus for integrin activation comes from inside the cell. The exact binding hierarchy of proteins is not totally solved but talin, kindlin and focal adhesion kinase (FAK) are known to be among the first proteins to bind to integrin cytoplasmic tails. This leads to separation of integrin tails and a conformational change. ECM ligands can bind to the integrin extracellular domain and other adaptor protein can be recruited to the intracellular tails (Danen 2005, Bouvard, Pouwels et al. 2013). Binding of ECM components, conformational changes, cytoplasmic tail separation and binding of talin is required for integrins to become fully active.

Active integrins have two main functions. They physically link the ECM to the cellular cytoskeleton creating forces between the ECM and cells, and they integrate signals from the ECM to the cell. Even though integrins lack kinase activity they regulate several signalling pathways by recruiting and activating kinases like FAK and Src inside the cell which in turn regulates cell proliferation, differentiation, survival, polarisation and migration (Hood and Cheresh 2002).

2.1.6 Integrins and cancer

Since integrins are important in regulating cell proliferation, migration and invasion, it is not surprising that misregulation of integrin expression or function is linked to cancer progression and metastasis. During metastasis integrins often mediate the binding of CTCs to the endothelial cell surface. Recent studies have also shown the importance of β1 integrin-mediated cell-ECM interactions when disseminated cancer cells colonize to distant organs. This interaction is followed by activation of FAK which enables the proliferation of tumour cells in an unfamiliar parenchyma (Barkan, Kleinman et al. 2008, Shibue and Weinberg 2009). In addition to cancer cells, integrins contribute to cancer progression by regulating the tumour microenvironment. Alterations in integrin activity, expression and function have been attributed to the cancer-supportive changes that occur in tumour-associated cells (e.g. in stromal fibroblasts and in the vascular endothelium). Cross-talk between integrins and GFRs is well-established. Integrin-GFR complex formation can cooperatively lead to enhanced activation of kinases like MAPK and AKT further inducing cellular proliferation (Shibue and Weinberg 2011).

Certain integrin heterodimers can be either up- or down-regulated in several cancer types. At least α5β1, α6β4, αvβ3, αvβ5 and αvβ6 integrins are known to be upregulated in malignant cells in different cancer types, where as α2β1 and α1β1 integrins can be up- or downregulated depending on the cancer (Table 1).

Table 1. Misregulation of integrin heterodimers in different tumour types. The most commonly up- or down-regulated integrin heterodimers are summarized. Adapted from Desgrosellier and Cheresh 2010.

Tumour type	Integrins expressed	Up- or down-regulated
Breast	α1β1, α6β4, αvβ3	up
Breast	α2β1	down
Prostate	αvβ3, α2β1	up
Ovarian	α4β1, αvβ3	up
Pancreatic	αvβ3	up
Melanoma	αvβ3, α5β1, α1β1, α2β1	up
Colon	αvβ6	up
Colon	α2β1	down
Non-small-cell lung carcinoma	α5β1	up
Glioblastoma	αvβ3 and αvβ5	up
Cervical	αvβ3 and αvβ6	up

Several studies have shown that αvβ3 and αvβ5 integrins are important for angiogenesis and tumour growth. These integrin receptors are overexpressed in both tumour cells and in angiogenic endothelial cells. In angiogenesis they support the formation of new vessels via different pathways (Weis and Cheresh 2011). Integrin αvβ3 is essential for angiogenesis mediated by basic fibroblast growth factor (bFGF) or tumour necrosis factor alpha (TNF-α), whereas αvβ5 integrin is needed for VEGF and transforming growth factor beta 1 (TGFβ1)-induced angiogenesis. Both of these αv heterodimers have also been implicated in metastasis. Integrin αvβ3 induces transendothelial migration (Bauer, Mierke et al. 2007), production of MMP-2 and promotes anchorage-independent growth (Baum, Hlushchuk et al. 2007). Integrin αvβ5, on the other hand, interacts with tyrosine kinase receptors (RTKs) such as epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR). The expression of αvβ5 and cytokine-mediated activation of IGFR are both required for cancer cell invasion and metastasis (Brooks, Klemke et al. 1997).

The β4 integrin subunit is different from other β subunits. It has a large cytoplasmic tail consisting of a kinase domain which can be phosphorylated by the Src family kinases. Phosphorylated β4 integrin further activates the PI3K

signalling pathway. Due to this β4-specific signalling mechanism, β4 integrin is unique among other integrin family members. β4 binds to laminin-5 and forms heterodimers only with the α6 subunit. Integrin α6β4 is overexpressed in many cancer types such as in breast and prostate cancers. In breast cancer the upregulation of α6β4 integrin is linked to basal-like breast cancers and it also correlates with disease aggressiveness. In the normal mammary gland, α6β4 integrin anchors the ECM to the actin cytoskeleton via hemidesmosomes. In invasive breast tumours cells lose their polarity, hemidesmosomes are disassembled and phosphorylated α6β4 is relocated to actin-rich protrusions mediating cancer cell migration and invasion. Moreover, several studies demonstrate that α6β4 integrin contributes to cancer progression by sustaining RTK signalling, especially EGFR, ERBB2 (HER2) and hepatocyte growth factor receptor (HGFR/Met) signalling. Due to this cross-talk between α6β4 integrin and RTKs, α6β4 integrin is implicated in generating the resistance to anti-EGFR-targeted therapy (Guo, Pylayeva et al. 2006, Yoshioka, Otero et al. 2013).

The β1 integrin subunit can pair with several α subunits to form a total of 12 different receptor combinations. These heterodimers can bind to several ECM components including collagens, laminins and fibronectin. This group of integrins demonstrate altered gene expression in several cancer types as shown in table 1. In general, β1 integrins are often overexpressed in cancer cells leading to enhanced FAK, ERK/MAPK, Src, AKT, and RAS downstream signalling. Through these pathways β1 integrin participates in regulating cancer cell proliferation, progression and metastasis in several cancers including melanoma, breast and prostate cancer (Koistinen and Heino 2002). In contrast, α2β1 heterodimer has been shown to function as a tumour suppressor at least in breast and prostate cancers. Integrin α2β1 is normally expressed on breast epithelium but in cancer cells its expression is decreased. Ramirez and colleagues showed recently that the deletion of α2β1 integrin leads to enhanced tumour cell intravasation *in vitro* and *in vivo* (Ramirez, Zhang et al. 2011).

Recently, an interesting β1 to β3 integrin switch was identified in breast cancer. First, Parvani and colleagues detected a compensatory increase in β3 integrin after β1 inhibition, which led to metastatic progression (Parvani, Galliher-Beckley et al. 2013). Truong and colleagues confirmed this with E-cadherin-positive TNBC cells (Truong, Xiong et al. 2014) where the loss of β1 and gain of β3 integrin expression relieved TGFβ inhibition.

There have been several pre-clinical studies for integrin antagonists but only a small number of these have entered clinical trials. Integrin inhibitors include peptides, organic small molecules or function-blocking antibodies which all function by interfering with ligand binding to the receptors. Anti-integrin compounds can inhibit cancer cell growth and interfere with the cancer

microenvironment. Currently, there are also ongoing studies to investigate combination treatments consisting of integrin-blocking agents together with chemotherapy or existing targeted therapies. By inhibiting cell adhesion cancer cells become more sensitive to radio-, chemo- or targeted therapy (Sethi, Ginsberg et al. 1999).

Few αvβ3/αvβ5 targeting drugs have entered phase I, phase II and even phase III clinical trials. Cilengitide, a cyclic arginine-glysine-aspartic acid (RGD)-peptide that selectively blocks αvβ3/αvβ5 integrin heterodimer function, was tested in combination with radiotherapy in a phase III clinical study for glioblastoma. However, this trial failed as cilengitide did not provide significant benefits to the patients (Stupp, Hegi et al. 2014). Cilengitide has also been investigated in phase II studies for the treatment of lung, breast, prostate, pancreatic and squamous cell cancers as well as in leukaemia, melanoma, and tumours of the brain and central nervous system (Beekman, Colevas et al. 2006, Nabors, Mikkelsen et al. 2007). Another inhibitor of αvβ3/αvβ5, antibody CTNO 95, has been the subject of phase I and II clinical trials (Mullamitha, Ton et al. 2007). Vitaxin/etaracizumab, a humanized antibody and αvβ3 specific antagonist, has already been tested in phase I and II clinical studies (Gutheil, Campbell et al. 2000) and is under evaluation in additional clinical trials.

Volociximab, a humanized antibody, inhibits α5β1 integrin activity and it has been tested in phase I and II clinical studies for solid tumours (Ricart, Tolcher et al. 2008). ATN-161, a small peptide that targets α5β1 integrin as well as other fibronectin-binding integrins (e.g. αvβ3), showed promising results in phase I clinical studies (Cianfrocca, Kimmel et al. 2006) and has now entered phase II clinical trials as both a monotherapy and in combination with chemotherapy (Thundimadathil 2012) (Table 2). Currently, there are no therapies targeting β4 integrin in clinical trials (Desgrosellier and Cheresh 2010).

Table 2. Integrin inhibitors in clinical trials. Summary of drugs targeting integrins and the state of clinical trials with these drugs.

Drug	Targeted integrin	Clinical status
Cilengitide	αvβ3, αvβ5	Phase II
CTNO 95	αvβ3, αvβ5	Phase I, II
Vitaxin/etaracizumab	αvβ3	Phase I, II
Volociximab	α5β1	Phase I, II
ATN-161	α5β1, αvβ3	Phase II

2.2 THE PI3K-AKT PATHWAY IN CANCER

2.2.1 AKT activation

AKT, also called PKB, is a serine/threonine kinase which regulates several cellular processes including apoptosis, proliferation, differentiation, migration and invasion. There are three highly homologous members in the AKT kinase family; AKT1, 2 and 3. These isomers share more than 80 % similarity at the amino acid level and consist of an N-terminal PH domain, a kinase domain and a C-terminal regulatory domain. AKT1 and AKT2 are ubiquitously expressed whereas AKT3 has a more restricted expression pattern. AKT3 is mainly expressed in the brain, kidney and heart and in a limited number of other tissues (Brodbeck, Cron et al. 1999, Fillmore, Wang et al. 2005). The activation of all AKT family members is believed to occur via a similar mechanism and is PI3K dependent. First, RTKs, G-protein-coupled receptors (GPCR) or integrins recruit PI3K to the plasma membrane where it phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP3). AKT kinase is recruited to the plasma membrane where it binds to PIP3 via its pleckstrin-homology (PH) domain. AKT-PIP3 interaction then changes the conformation of AKTs, allowing phosphoinositide-dependent kinase 1 (PDK1) to access and phosphorylate the AKT activation loop (T308 in AKT1, T309 in AKT2 and T305 in AKT3) leading to partial kinase activation. Full AKT kinase activation requires phosphorylation of another site (S473 in AKT1, S474 in AKT2, and S472 in AKT3) found in the carboxy-terminal hydrophobic motif of the kinase. Phosphorylation of these serine residues is mediated by the rapamycin-insensitive mTOR complex (mTORC2) or DNA-dependent protein kinase (DNA-PK). A fully-activated AKT kinase relocates to the cytoplasm where it participates in numerous cellular functions (Figure 3).

AKT inactivation can happen at different levels. Probably the best known inactivator of AKT is phosphatase and tensin homolog (PTEN) which is a dual protein/lipid phosphatase that dephosphorylates PIP3 to generate PIP2. Protein phosphatase 2A (PP2A), on the other hand, dephosphorylates the T308/T309/T305 site on AKT and PHLPP1/2 dephosphorylates the S473/S474/S472 site leading to inactivation of the AKT kinase (Hemmings and Restuccia 2015).

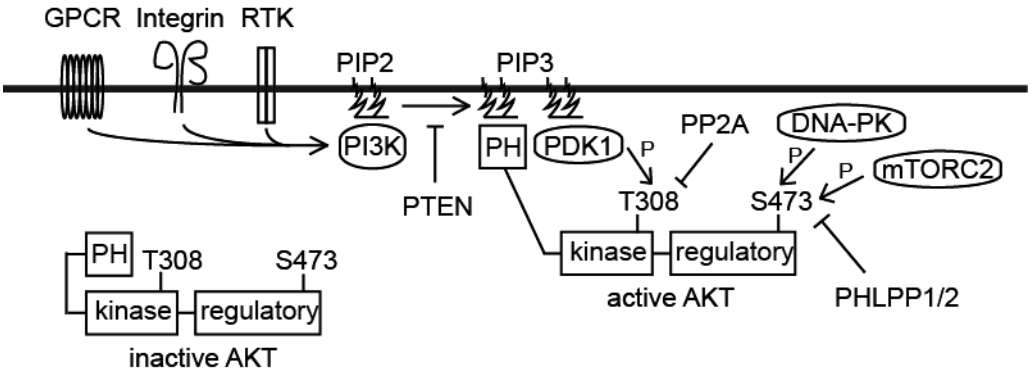

Figure 3. Activation of AKT kinases. Inactive AKT kinases are in a closed conformation in the cytoplasm. Following GPCR, integrin or RTK activation PI3K is activated which leads to phosphorylation PIP2, and PIP3 generation at the plasma membrane. Recruitment of AKT kinase to the membrane via its PH-domain results in an open kinase conformation. PDK1 then phosphorylates AKT at T308 which is followed by S473 phosphorylation by DNA-PK or mTORC2. PTEN dephosphorylates PIP3 to generate PIP2 leading to AKT inactivation. PP2A and PHLPP1/2 dephosphorylate T308 and S473, respectively to inactivate AKT. P stands for phosphorylation. GPCR, G-protein coupled receptor; RTK, receptor tyrosine kinase.

2.2.2 Downstream signalling of AKT kinases

AKT kinases participate in the regulation of many cellular processes by phosphorylating their target proteins. One of the best known roles of AKT kinases is in the regulation of apoptosis. AKT kinases inhibit apoptosis by phosphorylating and inactivating the pro-apoptotic factors procaspase-9 and bcl-2-associated death promoter. Additionally, AKT kinases inhibit transcription factor FOXO-dependent expression of pro-apoptotic genes like the FAS-L gene (TNFSF6) by phosphorylating and inactivating FOXO (Brunet, Bonni et al. 1999). On the other hand, AKT kinases induce apoptosis by positively regulating IκB kinase (IKK) which regulates the transcription of anti-apoptotic genes. AKT kinases can also regulate tumour suppressor p53 indirectly. AKT kinase phosphorylates mouse double minute 2 homolog (MDM2) which leads to its translocation into the nucleus, where MDM2 decreases p53 levels through degradation. Alternatively, AKT can phosphorylate transcription factor PHF20/TZP which results in its translocation to the cytoplasm, thereby suppressing p53 expression at the mRNA level (Nicholson and Anderson 2002).

AKT kinases are involved in cell cycle progression. AKT mediates cyclin D protein degradation by phosphorylating and inactivating glycogen synthase kinase 3β (GSK3β). In addition, AKT-mediated phosphorylation of cyclin-

dependent kinase (CDK) inhibitors results in translocation of these proteins into the cytoplasm where they cannot bind and inhibit the activity of cyclin/CDK complexes. AKT kinases also promote protein synthesis and angiogenesis by inhibiting complex formation between tuberous sclerosis 1 (TSC1) and 2 (TSC2). This results in induction of mTOR signalling which leads to enhanced protein synthesis and angiogenesis (Cheung and Testa 2013).

A role for AKT kinases has also been described in the regulation of cell migration and invasion. During embryogenesis, activated AKT kinase is essential for EMT-driven mesodermal cell migration (Grille, Bellacosa et al. 2003). During cancer cell migration and invasion, AKT kinases can induce cell motility by directly phosphorylating the IF protein vimentin (Zhu, Rosenblatt et al. 2011), a known marker of EMT which also stabilizes the EMT-promoting transcription factor Snail (Villagrasa, Diaz et al. 2012). In addition, active AKT promotes cell migration by regulating cytoskeletal reorganization and cell polarity at the cell leading edge. AKT activation directly modulates actin and this potentially affects cell migration. Through signalling crosstalk between AKT and RTKs such as VEGFR, AKT has been shown to positively regulate cell motility (Xue and Hemmings 2013).

2.2.3 AKT kinases in cancer

The PI3K-AKT pathway is frequently altered in human cancer. There are several mechanisms by which AKT signalling can be upregulated during cancer formation and progression. First, AKT kinases themselves can be amplified and overexpressed in cancer cells leading to constitutively active AKT kinases. AKT2 kinase is more frequently amplified than AKT1. AKT2 has been shown to be amplified and overexpressed at least in ovarian, breast and pancreatic carcinomas. The upregulation of AKT3 kinase in various cancers has also been demonstrated (Stahl, Sharma et al. 2004, Cristiano, Chan et al. 2006, Chin, Yoshida et al. 2014). Upregulation of AKT1 signalling has been mainly attributed to an activating mutation (E17K; substitution of glutamic acid with lysine residue at 17) in the AKT kinases PH domain rather than genetic amplification and/or protein overexpression. E17K mutation promotes the membrane localization and activation of AKT1 in cancer (Carpten, Faber et al. 2007).

Overexpression and/or mutations of upstream RTKs is one of the most common ways to activate AKT in cancer. In particular, EGFR, ERBB2, FGFR and Met amplifications have been reported in several cancer types resulting in PI3K and AKT activation. Downstream of RTKs is PI3K which consists of regulatory and catalytic subunits. The catalytic subunit, PIK3CA has been suggested to be an oncogene that is amplified in cancer. Also activating mutations in PIK3CA gene have been discovered. The regulatory subunit,

p85α, on the other hand, is a tumour suppressor and somatic mutations of this gene have been identified potentially leading to increased PI3K activity.

The tumour suppressor PTEN is known to be deleted or inhibited by inactivating mutations in a number of cancers. Recent studies have identified inositol polyphosphate 4-phosphatase B (INPP4B), a phosphatase responsible for PIP2 and PIP3 dephosphorylation, as a tumour suppressor. INPP4B has been shown to be mutated at least in ovarian, breast and prostate cancers leading to AKT hyperactivation (Gewinner, Wang et al. 2009, Kofuji, Kimura et al. 2015). The expression of another AKT inactivator PHLPP1/2 has been shown to be lost or downregulated in colorectal cancer (Liu, Weiss et al. 2009). Heat shock protein 90 (HSP90) is a molecular chaperone which assists in the folding and stabilization of many proteins for example EGFR, PI3K and AKT kinases. HSP90 overexpression in cancer results in prolonged activation of these proteins (Basso, Solit et al. 2002, Cheung and Testa 2013).

2.2.4 Distinct roles of AKT isoforms

Although AKT kinases have been extensively studied and there is lot of knowledge about the mechanisms of how AKT kinases and their downstream targets are regulated, surprisingly little is known about AKT isoform-specific functions. *In vivo,* knock-out mouse models have revealed non-overlapping activities for AKT isoforms in development. AKT1-depleted mice show growth retardation and perinatal lethality. AKT2-depleted mice develop an insulin-resistant diabetes-like phenotype, whereas AKT3-depleted mice have decreased brain size. In addition to developmental processes, AKT kinases have been reported to have distinct, even opposing functions in cancer. Most of the studies have been carried out in breast cancer models since in breast cancer AKT kinases are known to be hyperactivated. By using transgenic mouse models, xenograft models and studies in cell culture, researchers have defined specific roles for AKT1 and AKT2. In breast cancer, AKT1 kinase positively regulates primary tumour formation, but surprisingly inhibits lung metastasis. In other cancer types such as pancreatic and ovarian cancer, fibrosarcoma and soft tissue sarcoma, AKT1 has been reported to positively influence cancer cell motility and metastasis (Kim, Kim et al. 2001, Tanno, Tanno et al. 2001). In the case of AKT2, it inhibits breast cancer induction and growth but has an important role in metastatic dissemination (Hutchinson, Jin et al. 2004, Maroulakou, Oemler et al. 2007, Dillon, Marcotte et al. 2009). In breast and ovarian cancers, AKT2 has been shown to induce migration and invasion via upregulation of integrin signalling (Arboleda, Lyons et al. 2003). The AKT isoform-specific regulation of cancer growth and metastasis seems to be highly cell-type specific since in mouse embryonic fibroblasts (MEFs) AKT2, but not AKT1, inhibits cell migration. More recently AKT3 has been linked to breast cancer, especially to TNBC, where it regulates tumour growth independently of AKT1 and AKT2 (Chin, Yoshida et al. 2014).

2.3 THE MEK-ERK PATHWAY IN CANCER

2.3.1 Activation of the MEK-ERK pathway

Activation of ERK is a consequence of several phosphorylation events triggered by upstream kinases. Numerous extracellular signals, such as GFs and mitogens, can trigger the ERK activation cascade. The binding of GFs or mitogens to RTKs (e.g. EGFR) induces receptor dimerization and transphosphorylation. Activation of RTKs stimulates RAS activation; switching from inactive, guanosine diphosphate (GDP) bound state to the active guanosine triphosphate (GTP) bound state. This conformational change in RAS is followed by recruitment and binding of RAF to the membrane. In addition, the dimerization and phosphorylation of RAF is required for RAF activation. Dimeric, activated RAF in turn phosphorylates and activates MEK. Active MEK 1 and 2 activate ERK kinases by phosphorylating the threonine and tyrosine residues of ERK within the conserved T-X-Y sequence. When ERK is activated it can directly or indirectly phosphorylate several kinases, cytoskeletal molecules and transcription factors and in this way participate in the regulation of cell proliferation, differentiation, apoptosis and migration (Figure 4) (Marshall 1994, McKay and Morrison 2007). The specificity against a certain target depends on the strength of ERK activation, interactions with different scaffolding proteins and compartmentalisation (Lin, Wartmann et al. 1993, Ebisuya, Kondoh et al. 2005, Kang and Kim 2008).

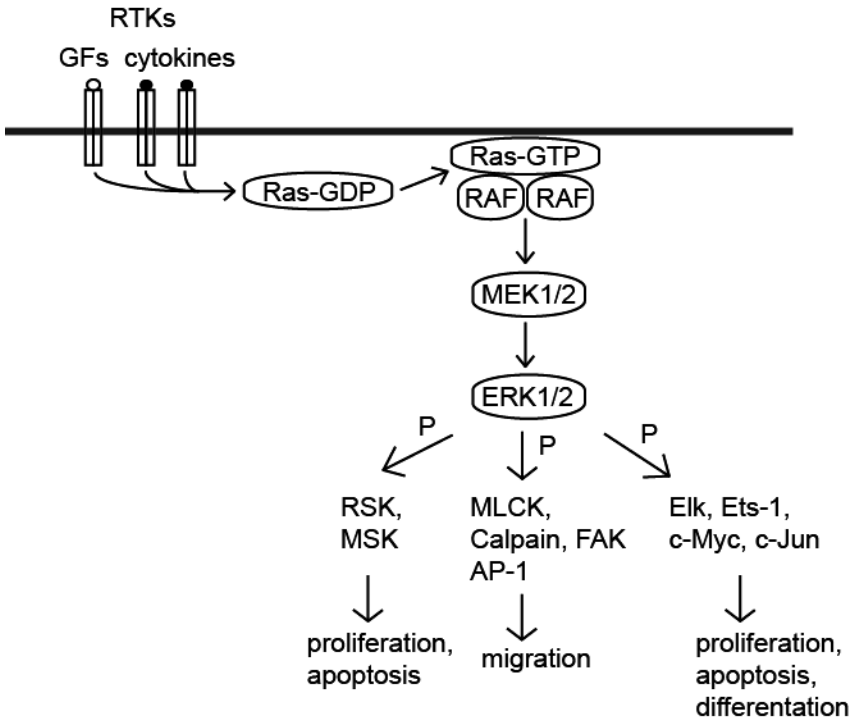

Figure 4. ERK kinase activation and downstream signalling. Binding of GFs or cytokines to RTKs leads to activation of RAS by switching RAS from GDP- to GTP-bound state. This is followed by RAF dimerization and activation at the plasma membrane. RAF further activates MEK and MEK phosphorylates and activates ERK. Several substrates of ERK are phosphorylated resulting in cell proliferation, apoptosis, migration and differentiation. P stands for phosphorylation. RSK, ribosomal s6 kinase; MSK, mitogen and stress activated protein kinase; MLCK, myosin light-chain kinase; FAK, focal adhesion kinase; AP-1, activator protein 1; Elk, Ets-1, c-Myc, c-Jun transcription factors.

Since the activation of ERK kinases is mediated via phosphorylation, the inactivation of ERK kinases is achieved through phosphatases. There are three types of phosphatases that dephosphorylate ERK kinases; serine/threonine phosphatases like PP2A, tyrosine phosphatases and dual-specificity phosphatases (DUSPs). MAP kinase phosphatases (MKPs) are a group of DUSPs that inactivate ERK. They bind to phosphorylated ERK and undergo a conformational change. This increases the catalytic activity of MKPs which leads to ERK dephosphorylation and inactivation (Camps, Nichols et al. 1998, Kondoh and Nishida 2007).

2.3.2 ERK kinases in cancer

In addition to the PI3K-AKT pathway, the RAF-MEK-ERK pathway is frequently deregulated in approximately 30 % of all human cancers. ERK is found to be hyperactivated in cancer mainly due to overexpression or activating mutations in RTKs, sustained paracrine or autocrine production of RTK-activating ligands or due to RAS or B-RAF mutations. Independently of ERK upstream signalling, ERK target molecules such as nuclear transcription factors myc and AP-1 can be amplified or deregulated in cancer. Thus, this pathway can be deregulated at several levels, emphasizing its importance in cancer. The specificity towards ERK activation comes from the RAS-RAF signalling axis (Dhillon, Hagan et al. 2007). Activating mutations in the RAS family of proteins (K-RAS, N-RAS and H-RAS) have been found in various tumour types for example in melanoma, pancreatic, lung and hepatic cancers. Mutations in oncogenic RAS GTPases are most often single base substitutions resulting in the stabilization of the active GTP-bound RAS and its downstream signalling. Activated RAS proteins interact directly with the RAF family of kinases (A-RAF, B-RAF and C-RAF, also called RAF-1) leading to constitutive ERK activation (McKay and Morrison 2007). B-RAF is the main isoform mutated and activated in cancer. In malignant melanoma B-RAF is mutated in more than 65 % of cases and also in other cancers but at a much lower frequency. In B-RAF. the substitution of valine with a glutamic acid at position 600 (V600E) leads to direct phosphorylation of MEK and hyperactivation of ERK. In addition, other residues of B-RAF are mutated (such as G465E, G465V and G595R) in tumours. Surprisingly, these mutations do not elevate B-RAF activity compared to the wild-type (wt) B-RAF. Nevertheless, these mutations still lead to ERK activation. This is explained by the ability of these mutants to activate RAF-1 which then activates MEK and ERK (Davies, Bignell et al. 2002, Wan, Garnett et al. 2004).

The two main isoforms of the ERK family are ERK1 and ERK2. They share approximately 80 % similarity and in general they function in a similar fashion. They are expressed in all tissues and cell types (Boulton, Nye et al. 1991), with ERK2 being the predominant isoform (Shaul and Seger 2007). ERK1-deficient mice develop normally; they are viable, fertile and normal in size. They have defects in thymocyte maturation and also increased synaptic plasticity in the striatum (Pages, Guerin et al. 1999, Mazzucchelli, Vantaggiato et al. 2002), whereas ERK2-deficient mice die early in development, indicating that ERK1 cannot fully compensate for the loss of ERK2 (Hatano, Mori et al. 2003, Saba-El-Leil, Vella et al. 2003, Yao, Li et al. 2003). Several studies describe preferential activation of only one ERK isoform (Papkoff, Chen et al. 1994, Sarbassov, Jones et al. 1997) and more recently, it was proposed that ERK1 and ERK2 isoforms could have different effects on cell proliferation, differentiation and gene expression that is dependent on the context (Li and Johnson 2006, Vantaggiato, Formentini et al. 2006). Similarly in cancer, it has

been shown that ERK1 and ERK2 regulate cancer cell motility differently. Radtke and colleagues revealed that in non-small cell lung carcinoma cell lines ERK2, but not ERK1, is needed for HGF-induced motility. They also showed that ERK2, but not ERK1, directly phosphorylates paxillin at serine 126 and in this way ERK2 regulates focal adhesion turnover (Radtke, Milanovic et al. 2013). In addition, it has been shown that ERK2, and not ERK1, controls cancer cell migration and invasion by negatively regulating the expression of Rab17 and liprin-β2 (von Thun, Birtwistle et al. 2012). To further demonstrate the ERK2 isoform-specific role in cancer progression and invasion Shin and colleagues showed that overexpression of ERK2 can induce EMT in non-transformed mammary epithelial cells (MCF10A cells) (Shin, Rath et al. 2010) and K-RAS G12D-activated ERK2 was found to be essential for 3D invasion of human pancreatic cancer cells via MMP-1 regulation (Botta, Reginato et al. 2012).

Taken together, there is increasing evidence showing the isoform-specific functions of ERK1 and ERK2 in cancer progression and cancer cell motility. Isoform-specific inhibitors against ERK could help to decrease cancer cell motility and probably diminish the side effects of current treatments.

2.4 EPITHELIAL-TO-MESENCHYMAL TRANSITION (EMT)

2.4.1 The EMT process

The process of EMT was first discovered in the early 1980s by Elizabeth Hay who observed this phenomenon in the primitive streak of chicken embryos (Hay 1995). Nowadays, EMT is studied extensively and it has been well characterized in different biological contexts. In EMT, epithelial cells undergo a series of events in which they lose their epithelial characteristics and gain mesenchymal properties. Epithelial cells lose their cell-cell and cell-ECM contacts, apical-basal polarity is disrupted, and the cells reorganize their actin cytoskeleton to become more motile, fibroblast-like mesenchymal cells. The loss of epithelial markers such as E-cadherin, zonula occludens protein-1 (ZO-1) and cytokeratins and gain of mesenchymal markers such as the intermediate filament vimentin, fibronectin and N-cadherin are typical events during EMT. Transcription factors including Snail, Slug, Twist-related protein 1 (TWIST) and Zinc finger E-box-binding homeobox 1 (ZEB1) are the main suppressors of E-cadherin and are therefore essential for EMT. Epithelial cells are surrounded by basal lamina and are joined together by special plasma membrane structures such as tight junctions, adherens junctions and desmosomes, which are lost during EMT (Figure 5) (Frixen, Behrens et al. 1991).

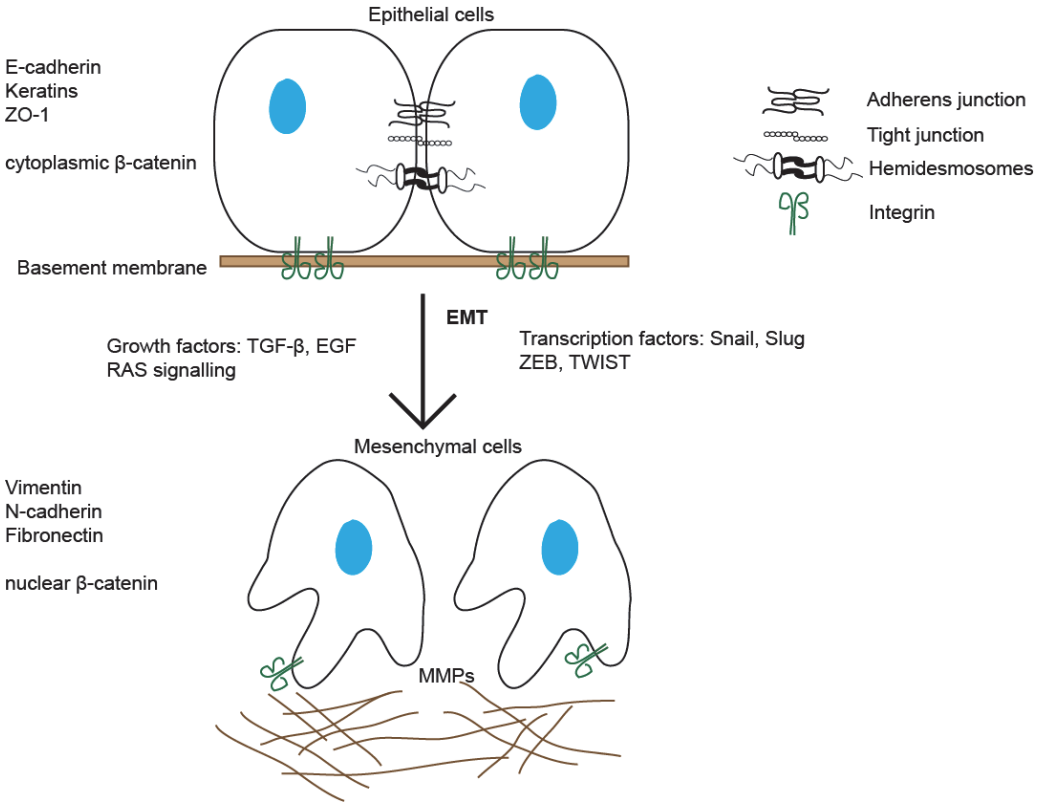

Figure 5. The process of EMT. Epithelial cells are bound together via different cell-cell junctions. They express epithelial markers like E-cadherin, keratins and ZO-1, and demonstrate a cytoplasmic localisation for β-catenin. Cells are surrounded by basement membrane and integrins are located on the basal side. EMT can be induced by GFs, RAS signalling or transcription factors. During EMT, epithelial cells lose their cell-cell contacts and start to express mesenchymal markers like vimentin, N-cadherin and fibronectin. In addition β-catenin is relocated to the nucleus and the basement membrane is degraded by MMPs and the ECM is modified.

The first step of EMT initiation is the disassembly of cell-cell contacts. In tight junctions ZO-1 binds to the cytoplasmic tails of claudin and occludin, two important components of tight junctions. During EMT ZO-1 is diffused from cell-cell contacts and these tight junction proteins are relocated and degraded. E-cadherin is a transmembrane glycoprotein that mediates cell-cell contacts in adherens junctions. It is a major determinant of EMT and becomes downregulated by EMT-inducing transcription factors. Additionally, E-cadherin is cleaved and degraded when adherens junctions are destabilized. In epithelial cells, cytoplasmic β-catenin and p120 catenin are known to bind E-cadherin and link E-cadherin to the cytoskeleton. During E-cadherin degradation β-catenin is released and translocated to the nucleus where it can

induce EMT by activating transcription factors that enhance Wnt signalling. The PAR, Crumbs and Scribble polarity complexes are responsible for apical-basal polarity in epithelial cells. During EMT the formation of these complexes is disrupted and expression of the principal components is repressed by EMT-inducers. Next, the epithelial cell shape has to be altered and the ECM around the cell has to be modified. During the transit of the epithelial cells towards a more mesenchymal phenotype, the cortical actin cytoskeleton is reorganised. At the cell edge, new actin-rich projections called lamellipodia and spike-like extensions termed filopodia are formed. Invadopodia, F-actin-rich matrix-degrading structures, are also formed. MMPs located in invadopodia have important roles in ECM degradation and modification during EMT and cell invasion. Rho GTPases are known to be responsible for actin dynamics and actin rearrangement during EMT. Most importantly, Rac1 and Cdc42 regulate the formation of lamellipodia and filopodia. RhoA, on the other hand, promotes actin stress fibre formation which is also an important factor in EMT induction (Lamouille, Xu et al. 2014).

The reverse process of EMT is mesenchymal-to-epithelial transition (MET). This occurs for example during metastasis. When mesenchymal cancer cells have metastasised to distant organs and they begin to colonize, cells may undergo MET. These cells have to regenerate the proliferative state, allow adhesion and form macrometastasis resembling the primary tumour (Kalluri and Weinberg 2009).

There are three types of EMT. Type 1 EMT occurs during embryogenesis and organ development. In the absence of EMT, the developmental process is halted at the early stages of embryogenesis. EMT occurring during tissue regeneration and wound healing and in pathological situations such as organ fibrosis has been designated as type 2 EMT. During wound healing keratinocytes go through partial EMT which allows them to migrate and close the wound while being loosely attached to neighbouring cells. Slug is needed for efficient wound closure. For example in human keratinocytes EGF induces ERK5 phosphorylation which leads to Slug expression and wound healing (Arnoux, Nassour et al. 2008). Type 3 EMT is closely linked to metastasis and occurs in cancer progression (Thiery, Acloque et al. 2009). The main focus of most EMT studies is on unravelling the detailed mechanisms of how EMT is initiated and what is its role in cancer metastasis. The other important topics explored by EMT researchers are the potential to target EMT for cancer treatment and to expand on the knowledge of EMT signature genes. Type 3 EMT will be discussed in more detail in the following chapter.

2.4.2 Regulation of EMT in cancer

EMT can be initiated by many different biological processes. These can be expression and activation of certain cell-surface proteins, activation of

transcription factors, re-organization of the cell cytoskeleton, expression of ECM-degrading enzymes and alterations in specific microRNA expression patterns. Several signalling pathways are implicated in EMT induction as well (Thiery 2002). There are several EMT-inducing GF type signals such as FGF, IGF, HGF, EGF, TGFβ and platelet-derived growth factor (PDGF) that are frequently generated by the tumour stroma. The best-known EMT-inducers are members of the TGFβ family of cytokines. TGFβ-mediated EMT is roughly divided into Smad-dependent and non-Smad activation. TGFβ binds to a heteromeric complex of type I and type II transmembrane serine-threonine kinase receptors. This binding activates the receptor complex and leads to phosphorylation and activation of Smad2 and Smad3, followed by trimer formation consisting of Smad2, Smad3 and Smad4. This trimer is then translocated into the nucleus where it associates with DNA-binding transcription factors in order to activate or repress target gene transcription. The Smad complex can also trigger the activity of EMT-related transcription factors. Distinct Smads, namely Smad 6 and 7, can inhibit the activation of TGFβ-activated Smads (Feng and Derynck 2005). TGFβ-dependent non-Smad signalling occurs through adaptor proteins or directly through the same receptor complex. This results in activation of the MEK-ERK kinase pathway, Rho GTPases and the PI3K-AKT pathway (Lamouille, Xu et al. 2014). These TGFβ-activated pathways are suggested to enhance TGFβ-Smad signalling and EMT induction. In addition, specific inhibitors against these pathways have been shown to block TGFβ-induced transformation (Bakin, Tomlinson et al. 2000, Bhowmick, Zent et al. 2001, Xie, Law et al. 2004, Cho, Baek et al. 2007).

RTKs can initiate either partial or full EMT independently of TGFβ. RTKs mainly act through PI3K-AKT, MEK-ERK, p38 MAPK and c-Jun N-terminal kinase (JNK) pathway and Src signalling and often lead to the expression of EMT-linked transcription factors such as Snail, Slug, FOS-related antigen 1 (FRA1) and TWIST (Lamouille, Xu et al. 2014). FGF-induced EMT in bladder carcinoma cells leads to α2β1 integrin and MMP13 expression instead of transcription factors and triggers destabilization of desmosomes (Valles, Boyer et al. 1996, Savagner, Yamada et al. 1997). Canonical Wnt signalling is induced by binding of Wnt ligands to a receptor called Frizzled. As a consequence GSK3β is inactivated, hence β-catenin phosphorylation, ubiquitination and degradation is prevented. β-catenin is relocated to the nucleus where it regulates gene expression of several EMT-inducing transcription factors such as Snail (Yook, Li et al. 2005) and ZEB1 (Sanchez-Tillo, de Barrios et al. 2011). In addition, the activation of canonical Wnt signalling has been shown to regulate Snail and Slug activity and stability. This induces EMT and has been linked to BRCA1 repression in a cohort of TNBC patients (Wu, Li et al. 2012). Moreover, Notch signalling has been implicated in EMT induction during development and in cancer progression. In breast cancer it has been shown that Notch can directly stimulate Slug expression and EMT (Leong, Niessen et al. 2007, Niessen, Fu et al. 2008). Notch, together with the

hypoxia pathway, has been shown to regulate EMT in different cancer cell lines (Sahlgren, Gustafsson et al. 2008). It has been suggested that gefitinib resistance in lung adenocarcinoma cells is due to an acquired EMT phenotype. Xie and colleagues show that at least in the PC9/AB2 lung cancer cell line EMT induction is Notch-1 dependent and the phenotype can be reversed by Notch silencing (Figure 6) (Xie, Zhang et al. 2012).

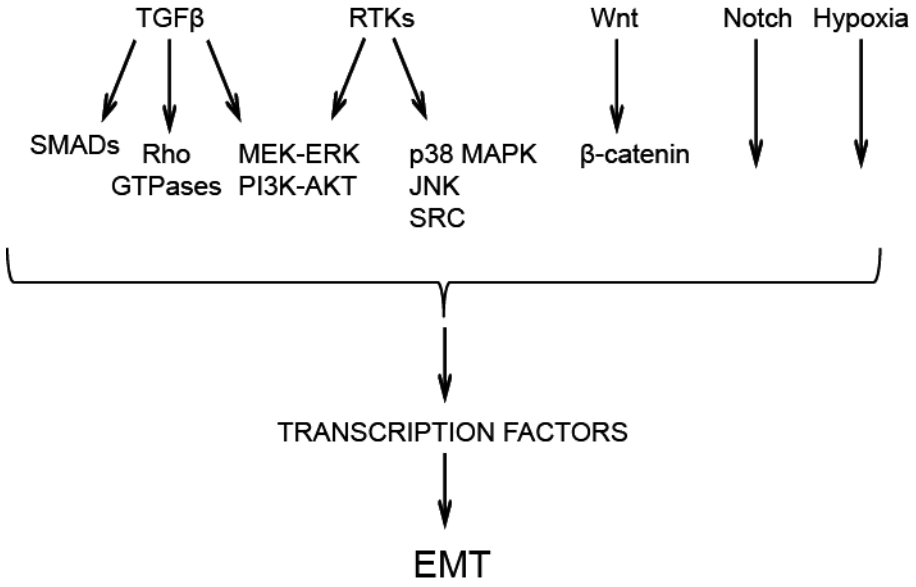

Figure 6. Overview of EMT-promoting signalling pathways. Activation of several signalling pathways can lead to the expression and activation of EMT-linked transcription factors which then induce EMT. The main reported pathways are shown in the figure.

Integrins play a role in cell adhesion and migration. Integrin complexes participate in signal transduction from the ECM via signalling mediators like integrin-linked kinase (ILK). During EMT there can be a shift in the repertoire of integrins expressed. For example Baldwin and colleagues showed recently that a complex formation between CD151 and α3β1 integrin inhibits Slug-mediated EMT and tumour growth in ovarian cancer (Baldwin, Hoff et al. 2014). Conversely, α5β1 integrin expression is induced in EMT and leads to increased cell adhesion to fibronectin and cell migration. Increased α1β1 and α2β1 integrin expression in EMT favours type I collagen signalling and leads to disruption of the E-cadherin–β-catenin complex (Shintani, Fukumoto et al. 2008). In addition, the expression and activation of several αv integrins, such as αvβ3 and αvβ5 is upregulated in EMT in a TGFβ-dependent manner. TGFβ signalling can activate αv integrins directly by phosphorylating the β subunit cytoplasmic tail or indirectly by modulating ECM production, remodelling or deposition (Mamuya and Duncan 2012). In addition it has been shown that

depletion of β5-integrin reduces cell-ECM adhesions and inhibits the TGFβ-induced EMT (Bianchi, Gervasi et al. 2010).

2.4.3 The intermediate filament vimentin

The cytoskeleton is composed of three main polymers; microtubules, actin filaments and IFs. In collaboration these three components of the cellular cytoskeleton control the cell shape, mechanics and cellular movement (Huber, Boire et al. 2015). However, there are clear distinctions between these main cytoskeletal polymers. Actin filaments and microtubules form polarized structures and support the directional movement of molecular motors, whereas IFs are not polarized and thus fail to support directional protein transport. The dynamics of assembly between the cytoskeletal polymers is different as well. IFs are elastic, less stiff than both microtubules and actin polymers and are able to effectively resist tensile forces (Fletcher and Mullins 2010). In general, IFs have cell- and tissue-type-specific expression patterns. There are approximately 70 different IF proteins characterized and these are divided into six different subfamilies. Acidic and basic keratins form type I and II IFs and are mainly found in epithelial cells. Vimentin belongs to a type III subfamily together with desmin, a muscle-specific IF. The type IV IFs consists of neurofilaments, type V of nuclear lamins and type VI IFs (e.g. nestin) are expressed in embryonic neurons. All IFs share a similar overall structure; they consist of a central rod domain flanked by an amino-terminal head and a carboxy-terminal tail domain (Satelli and Li 2011). Two monomers form a coiled-coil dimer which then forms tetramers and highly stable polymers. In general vimentin forms homopolymers but in addition, it is known to form heteropolymers in association with other type III or type IV IFs.

Vimentin is a key component of IFs in cells of mesenchymal origin, such as fibroblasts, endothelial cells and leukocytes (Franke, Hergt et al. 1987). Vimentin is expressed during normal development and in pathological conditions like wound healing and cancer metastasis. It is well accepted that vimentin is involved in several cellular processes and takes part in the formation of signalling and adaptor protein complexes. Therefore, vimentin has important roles in the regulation of adhesion, membrane traffic, inflammation, tissue aging and cell migration (Ivaska, Pallari et al. 2007).

In spite of widespread expression and important biological functions of vimentin, the vimentin knockout (VIM -/-) mouse appears to be surprisingly normal. It lacks overt phenotypes, develops normally and is fertile (Colucci-Guyon, Portier et al. 1994). This suggests that under normal physiological conditions, vimentin is not necessary for proper development. Subsequently, more detailed investigation of these mice has revealed more specific functions for vimentin especially in pathological situations. Eckes and colleagues showed that vimentin-deficient primary fibroblasts have a decreased ability to migrate

and are mechanically less stable (Eckes, Dogic et al. 1998). They also showed that wound healing in both embryonic and adult stages was impaired (Eckes, Colucci-Guyon et al. 2000). In addition, vimentin has a role in modulation of vasodilation (Terzi, Henrion et al. 1997), mechanotransduction of stress (Henrion, Terzi et al. 1997) and adult neurogenesis together with another IF protein glial fibrillary acidic protein (GFAP) (Larsson, Wilhelmsson et al. 2004). It has been shown that vimentin IFs are involved in lymphocyte adhesion and transmigration. VIM -/- lymphocytes have decreased homing ability to lymph nodes and spleen (Nieminen, Henttinen et al. 2006). The role of vimentin in tumorigenesis has been studied in a teratocarcinoma model. Embryonic stem cells (ES) were isolated from wt or VIM -/- mice and injected subcutaneously into wt or VIM -/- recipient mice. The absence of vimentin had no effect on proliferation or ES cell morphology *in vitro*, or on the ability of these cells to form teratocarcinomas *in vivo*. This suggests that at least in this model system, vimentin is not required for tumour formation and differentiation *in vivo* (Langa, Kress et al. 2000).

The regulation of vimentin function and dynamic polymer disassembly and assembly occurs via post-translational modifications including phosphorylation, sumoylation, O-GlcNAc modifications and citrullination. Phosphorylation is the most common post-translational modification of IFs. Indeed, several kinases can phosphorylate vimentin on different target sites *in vitro* but only a portion of these phosphorylation events have been confirmed *in vivo* and have a relevant biological role. Vimentin has a complicated phosphorylation pattern. During cytokinesis Rho kinase and protein kinase C (PKC) phosphorylate vimentin triggering a reorganisation of the vimentin network (Takai, Ogawara et al. 1996, Goto, Kosako et al. 1998). Later, additional kinases including Aurora-B, CDK1 and Polo-like kinase (Plk1) have been shown to phosphorylate vimentin during cell division (Goto, Yasui et al. 2003, Yamaguchi, Goto et al. 2005). In addition, protein kinase A (PKA) was shown to phosphorylate vimentin *in vivo* leading to disassembly of vimentin polymers (Eriksson, He et al. 2004). In smooth muscle cells, p21-activated kinase (PAK) phosphorylates vimentin and regulates the ratio of soluble to insoluble vimentin fractions (Tang, Bai et al. 2005, Li, Spinelli et al. 2006). In summary, the phosphorylation of vimentin has an important role in regulating vimentin structure and dynamic assembly but also impacts on the interaction between vimentin and intracellular structures and proteins.

2.4.4 The role of vimentin in EMT

The acquisition of migratory characteristics is one of the important hallmarks of EMT (Hanahan and Weinberg 2011). The intermediate filament vimentin is essential for migration and epithelial wound healing (Gilles, Polette et al. 1999, Rogel, Soni et al. 2011). For a long time vimentin was considered merely as a

marker of mesenchymal cells but it is now clear that it has a functional role in cancer initiation and processes like EMT and metastatic spread of cancer.

Mendez and colleagues have shown that vimentin regulates EMT-linked changes such as cell shape, adhesion dynamics and cell migration (Mendez, Kojima et al. 2010). During EMT, vimentin also promotes directional migration by interacting with Scrib which leads to stabilization of Scrib and correct polarization of the Golgi apparatus (Phua, Humbert et al. 2009). In addition, vimentin has a role in regulating cell adhesion and membrane traffic, processes that are important for cell migration (Ivaska, Pallari et al. 2007). Protein kinase C ε (PKCε) phosphorylation of vimentin at N-terminal serine residues results in enhanced cell motility. This occurs through integrin/PKCε vesicle recycling and trafficking to the cell membrane. PKCε-dependent phosphorylation of vimentin is needed for cargo release from these vesicles (Ivaska, Vuoriluoto et al. 2005). Recently, it was shown that the small GTPase Rab7a regulates vimentin phosphorylation and assembly (Cogli, Progida et al. 2013). In endothelial cells, vimentin is known to interact with β3 integrins. Vimentin regulates the strength of adhesion and interactions between cells and the ECM. Vimentin is recruited to focal adhesions in a β3 integrin- and plectin-dependent manner. This requires the microtubule cytoskeleton and its motor proteins (Bhattacharya, Gonzalez et al. 2009). In addition, plectin can function as a linker between the actin cytoskeleton and vimentin. More specifically, vimentin regulates the adhesion and morphology of the cell by interacting with actin stress fibre structures called transverse arcs in a process that also requires plectin (Jiu, Lehtimaki et al. 2015). In addition, actin and vimentin bind directly to each other and mediate cytoskeletal crosstalk and contribute to total cellular integrity (Esue, Carson et al. 2006). Moreover, vimentin regulates adhesion by directly binding to α2β1 integrin and further associates with filamin A, an important actin cross-linking protein (Kim, Nakamura et al. 2010).

Vimentin is known to be overexpressed in several human cancers such as prostate, breast and lung cancers as well as malignant melanoma, gastrointestinal tumours and tumours of the CNS. Furthermore, the expression of vimentin is often linked to metastatic disease and poor patient prognosis (Satelli and Li 2011). In lung cancer, vimentin depletion decreases the phosphorylation of guanine nucleotide exchange factor VAV2s and this reduces the activity of Rac1. VAV2-dependent Rac1 activation stabilizes FAK in vimentin- and FAK-positive focal complexes and further induces a more motile and invasive phenotype of lung cancer cells (Havel, Kline et al. 2015). In human breast cancer cell lines, complex formation between β-catenin and the transcription factor called T-cell factor (TCF) has been shown to transactivate vimentin expression and trigger cell migration and invasion (Gilles, Polette et al. 2003). In addition, it has been shown that vimentin is needed for Slug- or H-RAS-V12-mediated mesenchymal cell migration. By inducing Axl expression, vimentin regulates breast cancer cell migration in 2D and 3D environments and

participates in breast cancer cell extravasation to the lungs in mice (Vuoriluoto, Haugen et al. 2011).

In addition to roles as a scaffolding protein, vimentin actively participates in signalling pathways important in tumorigenesis. Vimentin has been shown to bind to 14-3-3 proteins and to inhibit their interaction with their target proteins. For example the interaction between phosphorylated vimentin and 14-3-3 proteins inhibits the assembly of the Raf/14-3-3 complex, leading to Raf activation (Tzivion, Luo et al. 2000). It is hypothesized that this is dependent on AKT-mediated phosphorylation of vimentin (Kidd, Shumaker et al. 2014). AKT1-dependent phosphorylation of vimentin at serine 39 allows vimentin to elude proteolysis and thus contributes to increased motility in soft tissue sarcoma (STS) cells (Zhu, Rosenblatt et al. 2011). In neurons, vimentin protects active ERK from dephosphorylation allowing it to signal longer (Perlson, Michaelevski et al. 2006). This has been shown to occur also in breast cancer cells (Virtakoivu, Mai et al. 2015). Thus, vimentin contributes to signalling pathways via several distinct mechanisms.

2.4.5 EMT inducers

Transcription factors are the master regulators of EMT. Their ability to directly or indirectly repress the expression of E-cadherin by binding to the E-cadherin gene (CDH1) promoter induces the transition from epithelial to mesenchymal morphology. Moreover, EMT-inducing transcription factors can simultaneously repress the expression of other junctional proteins such as claudins and desmosomal components which also participate in maintaining the epithelial phenotype. Following the discovery that the transcription factor Snail directly binds to and represses E-cadherin promoter activity (Batlle, Sancho et al. 2000, Cano, Perez-Moreno et al. 2000), many other transcription factors such as Slug (Hajra, Chen et al. 2002), ZEB1 and ZEB2 (Comijn, Berx et al. 2001, Eger, Aigner et al. 2005), Kruppel-like factor8 (KLF8) (Wang, Zheng et al. 2007), E47 (Perez-Moreno, Locascio et al. 2001) and Brachyury (Fernando, Litzinger et al. 2010) were identified to do the same. Snail and Slug belong to a family of Snail transcription factors that bind to DNA sequences called the E-box using their C-terminal zing-finger domains and repress the expression of epithelial genes. After binding to E-box motifs Snail and Slug recruit several other repressors, co-repressors and histone modifiers. In addition to suppression of epithelial genes, Snail has been shown to induce expression of mesenchymal genes for example fibronectin (Guaita, Puig et al. 2002, Stanisavljevic, Porta-de-la-Riva et al. 2011). ZEB1 and ZEB2 are members of the zinc finger E-box-binding homeobox family that bind directly to E-box elements of epithelial genes including CDH1. This is often followed by recruitment of a co-repressor called C-terminal-binding protein (CTBP) or in some cases recruitment of other additional molecules. ZEB expression is often triggered after Snail activation, which is not surprising since ZEB1 gene is a

direct target of Snail (Dave, Guaita-Esteruelas et al. 2011). In addition, other transcription factors have been identified which do not bind the E-cadherin promoter directly but can still induce EMT. These transcription factors include TWIST1 (Yang, Mani et al. 2004), forkhead box protein C2 (FOXC2) (Mani, Yang et al. 2007), E2-2 (Sobrado, Moreno-Bueno et al. 2009), Goosecoid (Hartwell, Muir et al. 2006), homeobox protein SIX1 (McCoy, Kawakami et al. 2009) and paired mesoderm homeobox protein 1 (PRRX1) (Ocana, Corcoles et al. 2012). TWIST1 and TWIST2 are believed to repress E-cadherin by association with other proteins such as polycomb complex protein 1 (BMI1) (Yang, Hsu et al. 2010). In cancer, under hypoxic conditions, hypoxia-inducible factor 1α (HIF 1α) drives TWIST expression which then promotes EMT (Lamouille, Xu et al. 2014). Furthermore, transcription factors like SRY box (SOX) can function synergistically with Snail or Slug to drive EMT. A good example of such regulation is SOX9 which in cooperation with Slug promotes EMT and tumorigenicity, and regulates the ability of breast cancer cells to metastasise (Guo, Keckesova et al. 2012). The hierarchy of these transcription factors is not known. They are expressed in a cell- and tissue-type-specific manner and studies often investigate transcription factors individually or in a limited set. However, in experimental models using different cell lines which co-express several EMT transcription factors, knockdown of just one transcription factors is sufficient to totally or partially block EMT (Olmeda, Montes et al. 2008, Spaderna, Schmalhofer et al. 2008, Casas, Kim et al. 2011).

In contrast, there are some examples of transcription factors which are important for maintaining epithelial cells. The expression of these transcription factors is downregulated in EMT and conversely upregulated during MET. Chakrabarti and colleagues showed that ETS-related transcription factor ELF5 can suppress EMT by repressing the expression of Slug (Chakrabarti, Hwang et al. 2012). Grainyhead-like-2 (GRHL2) transcription factor which is involved in epithelial morphogenesis has been shown to inhibit spontaneous EMT or TGFβ-induced EMT. It directly suppresses ZEB1 expression and enhances sensitivity to anoikis in breast cancer cells (Cieply, Riley et al. 2012, Cieply, Farris et al. 2013, Werner, Frey et al. 2013).

In addition to transcription factor-induced EMT, there is increasing evidence that other regulatory mechanisms like alternative splicing and expression of non-coding micro RNAs (miRNAs) can orchestrate EMT. The first example of a connection between EMT and alternative splicing was demonstrated with FGFR2 (Savagner, Valles et al. 1994, Foster, Kaplan et al. 1999). The expression pattern of FGFR isoforms has been shown to switch from FGFR2IIIb to FGFR1IIIc during TGFβ-induced EMT. After isoform switching, mesenchymal cells become sensitive to FGF2 signalling which is highly expressed in tumour tissues. Throughout EMT, many of the alterations in splicing are due to a downregulation of epithelial splicing regulatory protein 1 (ESRP) and 2. These two RNA-binding proteins control the splicing of several

gene transcripts. The downregulation of ESRP1 and 2 leads to an induction of mesenchymal protein isoforms and further contributes to cell motility, adhesion and signalling pathways (Warzecha, Jiang et al. 2010). A number of miRNAs have been associated with EMT. Non-coding miRNAs selectively bind to and inhibit mRNA translation or support mRNA degradation. Two main miRNA families, miR-200 and miR-34, have been shown to be important for EMT induction. The miR-200 family consists of five members, all of which are downregulated in mesenchymal cells. In epithelial differentiation miR-200 negatively regulates ZEB1 and ZEB2 expression in addition to downregulating the accessory epigenetic machinery, SUZ12 and BMI1 (Bracken, Gregory et al. 2008, Wellner, Schubert et al. 2009). The miR-34 family is also closely linked to EMT. The tumour suppressor p53 induces the expression of miR-34 family members which then inhibit EMT for example by downregulating RAS signalling (Kim, Roe et al. 2012) and repressing the expression of Snail (Kim, Kim et al. 2011).

2.4.6 Regulation of transcription factors

Post-translational modifications are known to regulate the localization and stability of transcription factors especially in the case of Snail. It is well known that GSK3β phosphorylates several transcription factors like p53 and Myc which leads to their nuclear export. GSK3β has been shown to phosphorylate Snail as well. There are two consensus motifs in Snail that contain serine residues targeted by GSK3β. Phosphorylation of the second motif, results in nuclear export of Snail. Furthermore, the phosphorylation of the first motif leads to binding of β-Trcp and degradation of Snail (Zhou, Deng et al. 2004). PDK1 has been shown to phosphorylate Snail at serine 11 (S11) leading to FBXO11 E3 ligase-mediated degradation of Snail (Zheng, Shen et al. 2014). Interestingly, this same site (S11) has been proposed to be phosphorylated by protein kinase A (PKA), resulting in stronger repression of E-cadherin expression (MacPherson, Molina et al. 2010). The same authors also proposed that independently of S11, the phosphorylation of serine 92 by casein kinase-2 would be required for E-cadherin suppression. In addition, PAK1-dependent phosphorylation of Snail at serine 246 leads to Snail accumulation in the nucleus which enhances Snail´s ability to suppress E-cadherin and occludin (Yang, Rayala et al. 2005). Lats2 interacts with Snail in the nucleus and directly phosphorylates it on threonine residue 203. This phosphorylation maintains Snail in the nucleus and enhances its stability (Zhang, Rodriguez-Aznar et al. 2012). In addition, active ERK2 has been shown to phosphorylate Snail and regulate its stability. This occurs via activation of the collagen I receptor discoidin domain receptor 2 (DDR2). Collagen I activates DDR2 which then phosphorylates ERK2 in a Src-dependent manner. ERK2 directly binds to and phosphorylates Snail on serine 82 and 104 sites. This leads to Snail nuclear accumulation and protection from degradation followed by production

of MT1-MMP. This DDR2-ERK2-pSnail-MT1-MMP pathway facilitates tumour cell invasion and sustains EMT (Figure 7) (Zhang, Corsa et al. 2013).

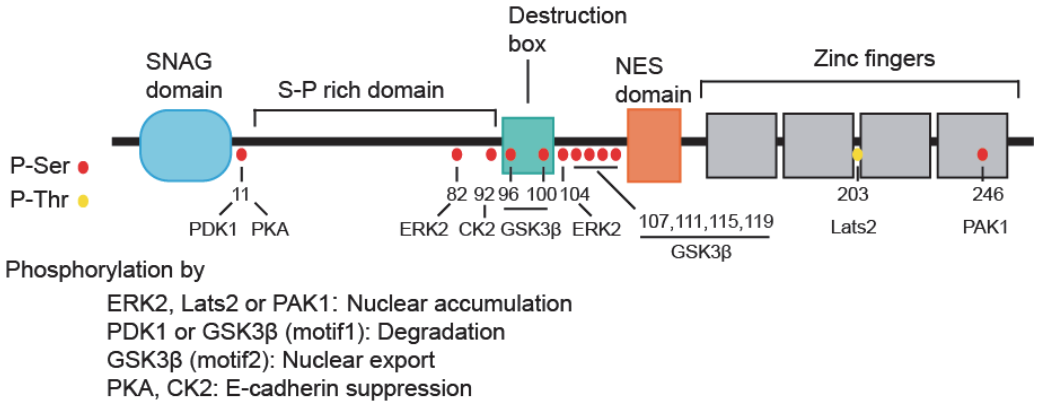

Figure 7. Snail structure and the regulation of Snail activity by phosphorylation. Snail consists of an N-terminal SNAG domain, an S-P rich region, a destruction box and NES domain and four C-terminal zinc finger domains. The main Snail phosphorylation sites are indicated in the figure; all of which, except one, are serine residues. Kinases phosphorylating these sites and the effect of each phosphorylation event are also shown in the figure.

Much less is known about the regulation of Slug by post-translational modifications. The first demonstration that Slug is phosphorylated in cells came from a study by Molina-Ortiz and colleagues. They showed that an overexpressed mouse Slug construct is phosphorylated at serine 4 and serine 88 residues in cells but only the phosphorylation of serine 4 has a functional role in EMT induction. This phosphorylation seemed to induce a stronger EMT phenotype than wt Slug (Molina-Ortiz, Villarejo et al. 2012). There is a GSK3β consensus phosphorylation sequence found in Slug which differs from the one found in Snail. Kim and colleagues suggest that phosphorylation of these serine residues would negatively regulate the stability of Slug and also cause cytosolic localization of Slug (Kim, Kim et al. 2012). Other studies have shown that GSK3β phosphorylates Slug *in vitro* at serine residues 92, 96, 100 and 104. GSK3β-mediated phosphorylation of Slug leads to Slug interaction with the carboxy-terminus of Hsc70-interacting protein (CHIP) and subsequent Slug degradation. The abolishment of GSK3β-mediated phosphorylation of Slug enhances lung adenocarcinoma cell migration and invasion and may induce metastasis *in vivo* (Kao, Wang et al. 2014). In addition Wu and colleagues showed that the canonical Wnt pathway regulates Slug expression, EMT induction and BRCA1 repression by inactivating GSK3β and inhibiting β-Trcp-mediated Slug degradation (Wu, Li et al. 2012). Recently, it was shown that ERK1 and ERK2 phosphorylate Slug at serine residues 87 and 104, but only the phosphorylation of serine 87 was implicated in EMT induction. The phosphorylation of Slug serine 87 was found to be required for proper induction

of vimentin and Axl expression during EMT initiation (Figure 8) (Virtakoivu, Mai et al. 2015).

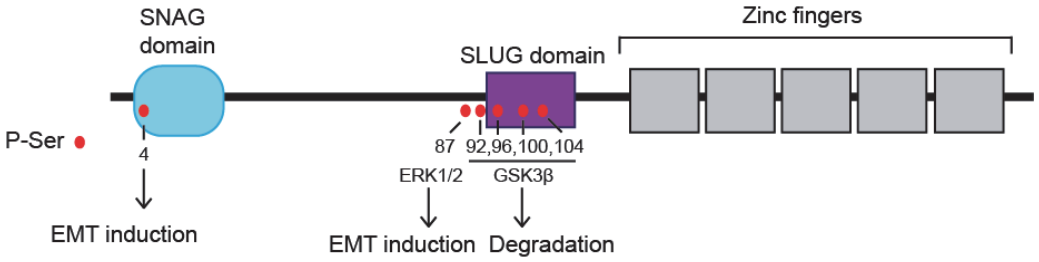

Figure 8. Slug structure and the regulation of Slug activity by phosphorylation. Slug consists of N-terminal SNAG and SLUG domains and five C-terminal zinc finger domains. The main phosphorylation sites are indicated in the figure. Kinases phosphorylating these sites and the effect of each phosphorylation event are also shown in the figure.

Phosphorylation-independent degradation of both Snail and Slug can occur via a Partner of Paired binding to these transcription factors during neural crest precursor formation (Vernon and LaBonne 2006, Lander, Nordin et al. 2011). In addition, MDM2 has been shown to target Slug for proteolytic degradation (Wang, Wang et al. 2009).

2.4.7 The receptor tyrosine kinase Axl

Axl belongs to a TAM (Axl, Tyro3 and Mer) family of RTKs. Despite distinct expression patterns and functions, all TAM family members can be activated by growth arrest-specific gene 6 (Gas6) ligand in a vitamin K-dependent manner. The Axl receptor can also be activated by dimerization with EGFR. Activation of Axl has been linked to several signalling pathways including the PI3K-AKT and RAS-Raf-ERK pathways. In this way Axl participates in the regulation of cell proliferation and survival. In addition, Axl plays a role in the suppression of the innate immune response (Linger, Keating et al. 2008, Linger, Keating et al. 2010). Axl is known to be overexpressed in many human cancers for example in lung, liver, prostate and breast cancers and in leukaemia, osteosarcoma and glioblastomas. The expression of Axl is induced by EMT and it is associated with aggressive disease state and poor prognosis. Axl has an important role in regulating the migration and progression of breast cancer cells (Gjerdrum, Tiron et al. 2010). In addition, Axl has been shown to regulate the function of breast cancer stem cells (Asiedu, Beauchamp-Perez et al. 2014). Meyer and colleagues showed that in TNBC cell lines the co-activation of Axl with EGFR family members Met and PDGF receptor (PDGFR) mediates resistance to EGFR-targeted inhibitors (Meyer, Miller et al. 2013). Moreover, in non-small cell lung carcinoma cell lines and in patients, resistance to both EGFR and PI3K pathway inhibitors was shown to be due to increased

Axl levels and activation (Zhang, Lee et al. 2012, Byers, Diao et al. 2013). These results suggest that Axl could be a potent new drug target for overcoming resistance to EGFR and PI3K inhibitors in breast and lung cancers.

3. AIMS OF THE STUDY

The general aim of this thesis work was to investigate how cancer cell migration and invasion are regulated. Specifically, the molecular mechanisms which regulate the transformation of cancer cells to a more motile and metastatic phenotype was studied.

Since EMT has been linked to cancer progression and metastasis, the regulation of EMT induction was also studied. In particular, the mechanistic role of the intermediate filament vimentin, a known marker of EMT, was investigated in this body of work.

Both PI3K-AKT and MEK-ERK pathway are known to be upregulated and activated in many human cancers but the cancer type specific regulation is still unclear. One of the aims was to delineate the isoform-specific functions of AKT and ERK kinases in prostate and breast cancer.

The specific aims of this study were:

- To investigate how different AKT isoforms regulate β1-integrin activity in prostate cancer
- To study the role of vimentin in EMT induction
- To study how vimentin specifically regulates ERK2 and Slug activity during EMT

4. MATERIALS AND METHODS

More detailed description of the methods and reagents is available in the original publications (I-II).

Experimental procedures

Method	Used in
Cell culture	(I,II)
DNA, siRNA and miRNA transfection	(I,II)
Cell adhesion assay	(I)
RTK array	(I)
ScanR analysis	(I)
Immunofluorescence microscopy	(I,II)
Western blotting (WB)	(I,II)
Flow cytometry (FACS)	(I,II)
Quantitative real-time polymerase chain reaction (qRT-PCR)	(I,II)
Proliferation assay	(I,II)
Cell migration assay	(I,II)
Cell invasion assay	(I,II)
Image analysis	(I,II)
Statistical analysis	(I,II)
DNA cloning	(II)
Site-directed mutagenesis	(II)
Immunoprecipitation (IP)	(II)
Immunohistochemistry and clinical studies	(II)
Sucrose gradient fractionations	(II)
Protein purification	(II)
Immunohistochemistry	(II)
In vitro kinase assay	(II)
Alkaline phosphatase protection assay	(II)
CAM assay	(II)
Luciferase reporter assay	(II)
In situ proximity ligation assay	(II)
STED	(II)

DNA constructs

DNA construct	Description	Used in
pcDNA3.1	Empty vector as a negative ctrl	(I)
pcDNA3_Hygro_HA_AKT2	Full-length AKT2	(I)
pcDNA3_vimentin	Full-length vimentin	(II)

pCMV	Empty vector as a negative ctrl	(II)
pCMV_MEK_ERK2	MEK_ERK2 fusion plasmid	(II)
CRU5-IRES-GFP	Empty vector as a negative ctrl	(II)
CRU5-IRES-GFP-Slug	Full-length Slug	(II)
p3XFLAG-CMV	Empty vector as a negative ctrl	(II)
p3XFLAG-CMV-Slug	Full-length Slug	(II)
pEGFP-C1	Empty vector as a negative ctrl	(II)
pEGFP-C1-Slug	Full-length Slug	(II)
pRL-TK	Renilla luciferase plasmid	(II)
pE-cad-571-Luc	E-cadherin promoter Luc reporter plasmid	(II)
pVIM-571-Luc	Vimentin promoter Luc reporter plasmid	(II)
Vim promoter GFP	GFP under vimentin promoter	(II)
pGEX-4T-1	GST expression vector	(II)

Point mutations were generated in the following constructs: CRU5-IRES-GFP-Slug, pEGFP-C1-Slug and p3XFLAG-CMV-Slug; -Slug_S87A, -Slug_S104A, -Slug_S87,104AA, Slug C161, 215A

siRNAs and pre-miRNAs

Name or target	**Description or cat. no and supplier**	**Used in**
GAPDH	Negative control, SI03571113, Qiagen	(I)
AKT1_5, #1	#SI00299145, Qiagen	(I)
AKT1_8, #2	#SI00287742, Qiagen	(I)
AKT1_10, #3	#SI02757244, Qiagen	(I)
AKT1_11, #4	#SI02758406, Qiagen	(I)
AKT2_5, #1	#SI00299166, Qiagen	(I)
AKT2_6, #1	#SI00299173, Qiagen	(I)
AKT3_2, #1	#SI00077581, Qiagen	(I)
Pre-miR negative control #1	AM17110, Ambion	(I)
Pre-miR-200a-3p	#PM10991, Ambion	(I)
Pre-miR-200b-3p	#PM10492, Ambion	(I)
Allstars negative control	Qiagen's negative control, mix of three non-effective siRNAs, Qiagen	(II)
ERK1 (MAPK3)	Mix of 4 siRNAs, L-003592-00, Dharmacon	(II)
ERK2 (MAPK1)	Mix of 4 siRNAs, L-003555-00, Dharmacon	(II)
ERK2 (MAPK1)	siERK2_11, J-003555-11, Dharmacon	(II)
ERK2 (MAPK1)	siERK2_12, J-003555-11, Dharmacon	(II)
Vimentin	Mix of 4 siRNAs, M-003551-01, Dharmacon	(II)
Vimentin	siVim_4, #SI00302190, Qiagen	(II)

SNAI2 (Slug)	Mix of 4 siRNAs, L-017386-00 Dharmacon	(II)
SNAI2 (Slug)	siSNAI2_5, SI03034416, Qiagen	(II)

Cell lines

All cell lines used in this thesis are of human origin, except MEF.

Cell line	Description	Used in
PC-3	Prostate cancer cells	(I)
MDA-MB-231	Breast adenocarcinoma cells	(II)
MCF10A	Nontumorigenic breast epithelial cells	(II,III)
MCF10AT	Premalignant breast epithelial cells	(II)
MCF7	Luminal-like breast cancer cells	(II)
HEK-293	Embryonic kidney cells	(II)
MEF	Mouse embryonic fibroblasts	(II)

The following stable cell lines were generated or kindly provided:

MEF: wt and ko VIM

MCF10A: CRU5-IRES-GFP, CRU5-IRES-GFP-Slug_wt, -Slug_S87A, -Slug_S104A, -Slug_S87,104AA

MDA-MB-231: shLuc, shAxl (Prof. J. Lorenz, University of Bergen)

Antibodies

Antigen	**Species**	**Description and supplier**	**Used in**
AKT1	Mouse	#2967S, Cell Signaling	(I)
AKT2	Rabbit	#2962, Cell Signaling	(I)
AKT3	Rabbit	07-383, Millipore Upstate	(I)
AKT	Rabbit	#9272, Cell Signaling	(I)
p-AKT	Rabbit	Ser308, #9275S, Cell signaling	(I)
p-AKT	Rabbit	Ser473, #9271S, Cell signaling	(I)
p-GSK 3β	Rabbit	Ser9, #9336, Cell signaling	(I)
EGFR	Rabbit	#2232S, Cell signaling	(I)
p-EGFR	Rabbit	Tyr 1068, #2234, Cell signaling	(I)
Met	Mouse	(L41G3), Cell signaling	(I)
p-Met	Rabbit	Tyr1234/1235, Cell signaling	(I)
Vinculin	Mouse	V9131, Sigma-Aldrich	(I)
β-Tubulin	Mouse	12G10, DHSB, Iowa	(I,II)
β-Actin	Mouse	JLA20, DHSB, Iowa	(I,II)
GAPDH	Mouse	5G4, HyTest	(I,II)
β1-integrin	Mouse	K20, Beckman Coultier	(I)

β1-integrin	Mouse	12G10, Abcam	(I)
β1-integrin	Rat	9EG7, BD Pharmingen	(I)
β1-integrin	Mouse	Mab13, BD Pharmingen	(I)
β1-integrin	Mouse	MAB2252, BD Transduction Lab	(I,II)
p44/42	Rabbit	ERK1/2, Cell signaling	(II)
p-p44/42	Rabbit	Thr202/Tyr204, Cell signnaling	(II)
Vimentin	Mouse	V9, sc-6260, Santa Cruz	(II)
Vimentin	Rabbit	HPA001762 clone, V6630, Sigma-Aldrich	(II)
Axl	Rabbit	H-124, SC-20741, Santa Cruz	(II)
Axl	Mouse	(Z49M), sc-73719, Santa Cruz	(II)
Axl	Goat	AF154, R&D Systems	(II)
Lamin A/C	Mouse	sc-7292, Santa Cruz	(II)
Keratin-8	Rat	Hybridoma bank	(II)
GST	Mouse	A00865, GenScript	(II)
Slug	Rabbit	C19G7, #9585, Cell signaling	(II)
pSlug S87	Rabbit	Ser 87	(II)
Snail	Rat	SN9H2, # 4719, Cell signaling	(II)

Reagents and chemicals

Compound	**Supplier**	**Used in**
HiPerfect	Qiagen	(I-II)
Lipofectamine 2000	Invitrogen	(I-II)
OptiMem	Invitrogen	(I-II)
DAPI	Sigma-Aldrich	(I-II)
Phalloidin-AF 488/647	Molecular Probes	(I-II)
Mowiol	Calbiochem	(I-II)
DMSO	YA-Kemia	(I)
PAN-AKT inhibitor (Triciribine)	Sigma-Aldrich	(I)
WST-1	Roche	(I-II)
RT-PCR primers and probes	Roche	(I-II)
Fibronectin	Calbiochem	(I)
Collagen	Sigma-Aldrich	(I)
TGFβ	R&D Systems	(II)
EGF growth factor	Sigma-Aldrich	(II)
U0126	Sigma-Aldrich	(II)
Recombinant active ERK1	ProQinase	(II)
Recombinant inactive ERK2	ProQinase	(II)
Recombinant active ERK2	ProQinase	(II)
Recombinant active Akt1	ProQinase	(II)
ATP- γ-P32	Sigma-Aldrich	(II)
Alkaline phosphatase	Promega	(II)

Vimentin	Cytoskeleton Inc.	(II)
GST-Vimentin	Spring Bio	(II)
Actin	Cytoskeleton Inc.	(II)

5. RESULTS

5.1 AKT ISOFORM-SPECIFIC REGULATION OF β1-INTEGRIN ACTIVITY AND CELL MOTILITY IN PROSTATE CANCER (I)

5.1.1 AKT kinases regulate β1-integrin activity and cell adhesion

In prostate cancer the PTEN tumour suppressor gene is often mutated and inactivated. This leads to the activation of the PI3K-pathway in which AKT kinases are the main downstream effectors (Majumder and Sellers 2005). A cell-spot microarray RNA interference screen was performed to identify β1-integrin activity regulators (Pellinen, Rantala et al. 2012). The screen was performed on 8 different prostate cell lines (6 prostate cancer cell lines, 1 primary prostate epithelial cell line and 1 primary prostate stromal cell line). The AKT1 isoform was identified as one of the strongest hits. AKT1 silencing upregulated β1-integrin activity in all of the prostate cancer cell lines tested (I, Fig. 1A). This is demonstrated in the microscopy images taken from control- or AKT1-silenced PC3 cells (I, Fig. 1B). We chose the PC3 cell line for further validation of results since these cells are highly invasive and migratory. First, we treated the cells with a pan-AKT inhibitor (AKTi) and noted that inhibition of AKT specifically reduced the levels of phosphorylated AKT (I, Fig. 2A) but did not significantly inhibit cell proliferation (I, Fig. 2B). Flow cytometric (FACS) analysis of β1-integrin cell surface levels using either 12G10 (anti-active β1-integrin antibody) or K20 (anti-total β1-integrin antibody) demonstrated that AKTi-treated cells had significantly more active β1-integrin on the cell surface compared to control cells (I, Fig. 2C). This was not due to induction of total β1-integrin levels since they were not altered (I, Fig. 1A and 2C). Also, we could see that the AKTi-treated cells displayed improved adhesion to collagen which corresponded to increased β1-integrin activity (I, Fig. 2D).

There are three members of the AKT kinase family. In breast and ovarian cancer, AKT isoforms have been shown to have specific, even opposite roles in regulating cell migration and invasion (Arboleda, Lyons et al. 2003, Irie, Pearline et al. 2005, Yoeli-Lerner, Yiu et al. 2005, Meng, Xia et al. 2006). To study if the upregulation of β1-integrin activity in AKTi-treated cells was specifically due to an individual AKT isoform, we used siRNA-mediated silencing of the distinct isoforms. The silencing of AKT isoforms specifically reduced the expression of each isoform (I, Fig. 3A, S1A) but had no effect on proliferation (I, Fig. 3B). FACS analysis of β1-integrin activity and total protein levels in AKT-silenced PC3 cells showed that both AKT1 and AKT2 silencing promoted β1-integrin activity whereas AKT3 silencing had no effect (I Fig. 3C, S1B). To further investigate the levels of active and total β1-integrin in cells, we fixed, permeabilized and stained adherent AKT1- or AKT2-silenced PC3 cells with 12G10 and K20 antibodies. Cells were imaged with automated ScanR

microscopy and more than 5000 cells per treatment were analysed. Silencing of both AKT1 and AKT2 significantly increased active β1-integrin levels. As we observed significantly enhanced adhesion to collagen following AKT inhibition (AKTi-treated cells), we wanted to further study the state of focal adhesions in AKT-silenced PC3 cells. In agreement with improved cell-ECM adhesions following AKT inhibition, we observed an increase in the number and size of vinculin-positive focal adhesions in AKT1- and AKT2- silenced cells (I, Fig. 4A-C).

Taken together, in PC3 cells, AKT1 and AKT2 function as negative regulators of β1-integrin activity without altering total β1-integrin levels. AKT1 and AKT2 also negatively regulate the amount and size of focal adhesions in PC3 cells.

5.1.2 Silencing of AKT1 and AKT2 inhibits cell migration and invasion

Integrins are known to participate in the regulation of cell migration. Active integrins cluster together, bind to ECM and the actin cytoskeleton to generate traction forces needed for cell migration. Since AKT1 and AKT2 silencing induced integrin activation, it prompted us to investigate how AKT silencing influenced PC3 cell migration. We used two different models to study cell migration. We first plated AKT-silenced cells on plastic and followed their migration with time-lapse imaging for 21 h. With AKT1 silencing we saw a small but significant increase in the total distance migrated (path length) (I, Fig. 5A) and a more notable increase in the persistence of cell migration (I, Fig. 5B-C). In contrast, AKT2 silencing did not have any effect on cell migration on plastic (I, Fig. 5A-C). We then studied migration on fibroblast-produced cell-derived matrices [CDMs; (Even-Ram and Yamada 2005)] where cells are in a more *in vivo*-like three-dimensional environment. Interestingly, on CDMs AKT2 silencing led to a longer migration distance and more persistent cell migration but AKT1 silencing had no significant effect (I, Fig. 5D-F). AKT3 silencing had no effect on migration distance on plastic or on CDMs but surprisingly AKT3 silencing induced significantly more persistent migration on plastic when compared to control cells (I, S2A-D).

As the AKT2 kinase regulated cell migration on CDMs, we wanted to further study the motility of AKT1-, AKT2- or AKT3-silenced PC3 cells using three-dimensional invasion assays. For this purpose we plated cells on the bottom of Ibidi μ-slide wells and followed cell invasion through the overlying matrigel towards an increasing serum gradient for 4 days. Fixed and actin-stained cells were imaged with confocal imaging. Both AKT1 and AKT2 silencing significantly increased cell invasion and importantly this effect was abolished by adding anti-β1 integrin function blocking antibody to the invading cells (I, Fig. 6A-B). The silencing of AKT3 had no influence on cell invasion (I, Fig. 6A).

In conclusion, AKT1 and AKT2 both regulate PC3 cell migration but in different environments. AKT1 silencing induces cell migration on plastic whereas AKT2 silencing appears to regulate cell migration on CDMs. AKT1 and AKT2 also negatively regulate PC3 cell invasion through matrigel.

5.1.3 AKT1 and AKT2 regulate β1-integrin activity via different mechanisms

We wanted to investigate in more detail how AKT kinases regulate β1-integrin activity and PC3 cell migration and invasion. We were also interested in investigating whether the underlying mechanisms mediating cell migration and invasion were distinct between AKT1 and AKT2 isoforms. Iliopoulos and colleagues showed that AKT isoforms differentially regulate the abundance of miR-200 family members in breast epithelial cells (Iliopoulos, Polytarchou et al. 2009). They showed that activated AKT2 isoform reduces the levels of miR-200 family members. This prompted us to study the levels of miR-200 family members, miR-200a and miR-200b, in PC3 cells. Using real-time reverse transcription PCR (qRT-PCR) we found that AKT2 silencing increased the levels of both miR-200a and b and AKT1 silencing only modestly decreased the levels of miR-200a and b (I, Fig. 7A). Next, we studied the effect of AKT2 overexpression and found that miR-200a was downregulated whereas miR-200b levels remained unaffected (I, Fig. 7B). Although miR-200 family members have been shown to negatively regulate EMT by suppressing the expression of transcription factors ZEB1 and ZEB2, there are several studies indicating that they can also induce breast cancer metastasis (Dykxhoorn, Wu et al. 2009). Additionally, miR-200 has been shown to be upregulated in invasive melanoma cells (Elson-Schwab, Lorentzen et al. 2010). To investigate whether AKT2 can regulate β1-integrin activity via regulation of miR-200a and b, we transfected PC3 cells with pre-miR-200a or pre-miR-200b and performed FACS analysis of β1-integrin activity. Indeed, we saw that pre-miR-200a and pre-miR-200b induced β1-integrin activity but did not affect total β1-integrin levels (I, Fig. 7C). In addition to this, we observed that miR-200a increased the size and number of vinculin-positive focal adhesions (I. Fig. 7D). Correspondingly, overexpression of miR-200a enhanced PC3 cell migration and modestly affected PC3 invasiveness (I, Fig. 7E-F). Taken together, in PC3 cells, AKT2 regulates β1-integrin activity and cell migration by regulating miR-200 levels.

Integrins are known to crosstalk with RTKs. Many RTKs like EGFR and Met have been shown to positively regulate integrins (Moro, Venturino et al. 1998, Ivaska and Heino 2011). In accordance with functional receptor crosstalk, integrin-dependent adhesion has been connected to ligand-independent RTK activation (Mitra, Sawada et al. 2011). Negative feedback loops effectively regulate RTK signalling in cells. Chandarlapaty and colleagues demonstrated that AKT inhibition relieves a feedback pathway involved in the suppression of

numerous RTKs in cancer cells (Chandarlapaty, Sawai et al. 2011). In line with this published data, we detected upregulation of phosphorylated RTKs after AKT1 silencing in PC3 cells (I, Fig. 8A). To compare the levels of active RTKs in AKT1- or AKT2-silenced cells we used an anti-phosphotyrosine receptor antibody array which included 42 active RTKs. AKT1 silencing increased the phosphorylation of several RTKs, with a prominent effect on EGFR and Met phosphorylation (I, Fig. 8A-B). These two RTKs were also highly phosphorylated in PC3 cells under normal conditions (I, S4A). In contrast, the silencing of AKT2, except for a strong induction of PDGFR α, did not influence the phosphorylation of all other RTKs tested (I, Fig. 8A-B). The observed upregulation of EGFR and Met phosphorylation upon AKT1 silencing was further confirmed with western blotting by using the phospho-specific antibodies against EGFR and Met (I, S4B). This negative correlation between AKT1 and Met was particularly interesting and was additionally observed in clinical samples. In silico meta-analysis of 208 prostate cancer samples and 147 skin tumours (Kilpinen, Autio et al. 2008), revealed a strong inverse relationship between AKT1 mRNA and Met mRNA levels specifically in prostate, but not in skin cancers (I, Fig. 8C). In contrast to AKT1, AKT2 mRNA levels correlated to some degree with Met mRNA levels in both cancer types (I, Fig. 8D). In conclusion, we observed a negative feedback loop between AKT1 and RTKs, mainly EGFR and Met. Upon AKT1 silencing, AKT1-dependent suppression of EGFR and Met was relieved and EGFR and Met phosphorylation was increased more than two-fold. Furthermore, this anti-correlative link was also seen at the mRNA level in clinical prostate tumour samples, but not in skin tumour samples.

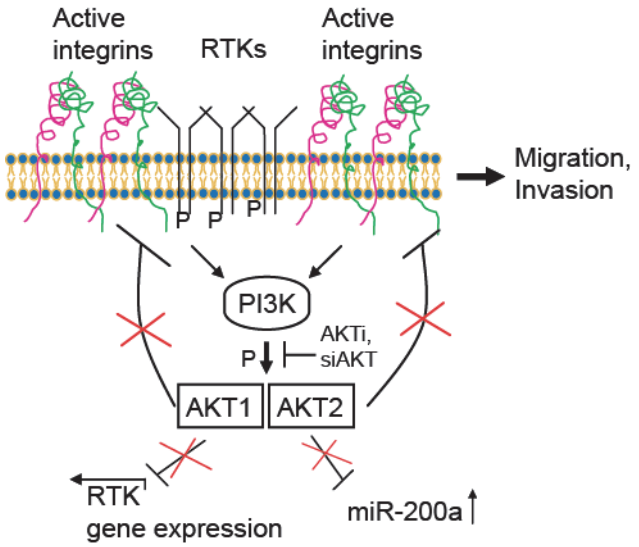

Figure 9. Model for AKT1- and AKT2-dependent regulation of β1-integrin activity and prostate cancer cell motility. The silencing of AKT1 relieves a negative feedback suppression of RTK expression and activation, leading to β1-integrin activation whereas the silencing of AKT2 induces miR-200a expression which contributes to β1-integrin activation. Inhibition of AKT1 and AKT2 results in increased β1-integrin activity and contributes to enhanced prostate cancer cell migration and invasion. Adapted from (Virtakoivu, Pellinen et al. 2012).

5.2 VIMENTIN-ERK-DEPENDENT REGULATION OF EMT VIA SLUG (II)

5.2.1 Vimentin, ERK and Slug are coexpressed in breast cancer and regulate breast cancer cell motility

Vimentin, Slug and ERK kinases are known to be important in EMT and breast cancer invasion. We and others have shown that in a collection of breast cancer tissue samples (356 samples), vimentin and slug are expressed specifically in TNBC (164 samples), the most aggressive breast cancer subtype (II, Fig 1A) (Karihtala, Auvinen et al. 2013). When we assessed the location of these proteins in the triple-negative tissue samples, we noticed pronounced expression of Slug, ERK and active, phosphorylated ERK (pERK) at the tumour periphery whereas vimentin was expressed all over the tumour (II, Fig 1B). In addition, in an orthotopic MDA-MB-231 xenograft model, we observed strong vimentin staining in spontaneously formed lymph node metastases compared to the primary tumour (II, Fig 1E).

As we detected vimentin, Slug and ERK at the invasive front of tumours, we wanted to further investigate the role of these proteins in the motility of TNBC

MDA-MB-231 cells. In 2D, depletion of Slug, vimentin, ERK1 and ERK2 resulted in significant inhibition of MDA-MB-231 cell migration (II, Fig. 2C, and S1A) (Vuoriluoto, Haugen et al. 2011). Slug, vimentin and ERK2, but not ERK1, silencing also significantly decreased the invasiveness of MDA-MB-231 cells through a laminin-rich extracellular matrix (matrigel) (II, Fig. 1D, and S1A). Importantly, these effects on breast cancer cell migration and invasion were not due to defects in cell proliferation (II, Suppl. Fig. S1B). Since we observed that ERK2, and not ERK1, silencing inhibited cell invasion, which is in line with previously reported results (von Thun, Birtwistle et al. 2012, Radtke, Milanovic et al. 2013), we focused only on the ERK2 isoform and on how it regulates EMT induction. Collectively, our results demonstrate coexpression of vimentin, Slug and ERK2 in the majority of TNBCs and highlight these proteins as important regulators of migration and invasion in the TNBC MDA-MB-231 cell line.

5.2.2 Vimentin, ERK2 and Slug are co-regulated

Recent studies have shown a functional role for vimentin during EMT but the detailed mechanisms are still missing. Downstream of TGFβ or active RAS signalling vimentin supports EMT induction (Vuoriluoto, Haugen et al. 2011). We detected strong vimentin expression in H-RAS transformed MCF10AT cells as compared to nontransformed MCF10A cells. Furthermore, the levels of active ERK were increased in MCF10AT cells. To study in more detail the connection between vimentin and ERK expression, we used a common EMT model where EMT is induced in MCF10A cells with TGFβ treatment. Firstly, we observed a correlation between vimentin and ERK activation in this model system and when we silenced vimentin the levels of active ERK decreased (II, Fig. 2B). This occurred also in vimentin-positive MDA-MB-231 cells (II, Suppl. Fig S2A). To further validate our data, we generated immortalized wt and VIM -/- mouse embryonic fibroblasts (MEFs) and studied the levels of total and active ERK in these cells. We saw that in Vim KO MEFs the phosphorylation of ERK was almost totally abolished whereas total ERK levels were only slightly decreased (II, Fig. 2C). ERK phosphorylation was rescued with re-expression of wt vimentin in Vim KO MEFs (II, Fig. 2D). Together our results suggest that vimentin expression may be needed for stabilization of active ERK.

To study the detailed mechanism behind vimentin-dependent ERK regulation, we investigated the localization of vimentin and active ERK in cells. Interestingly, using super-resolution stimulated emission depleted (STED) microscopy we saw that a portion of active ERK was located on vimentin filaments and that this pERK signal was reduced after treating these cells with the ERK inhibitor U0126 (II, Fig 2E). In addition, by using the *in situ* proximity ligation assay (PLA), we detected a significant PLA signal between vimentin and pERK that was completely lost after U0126 treatment (II, Fig 2F). To elucidate if the vimentin-ERK interaction was direct and/or phosphorylation-

dependent, we performed *in vitro* binding assays with pure recombinant proteins. In a GST-vimentin pulldown assay, we saw specific interaction between pERK and vimentin (II, Fig 2G). Vimentin-ERK interaction was further confirmed by first pulling down pERK and detecting its interaction with recombinant vimentin (II, Fig 2H). In summary, we demonstrate association between vimentin expression and active ERK in different model systems in cells. We also provide evidence that vimentin and ERK interaction occurs via direct binding.

The RAS-RAF pathway is the main activator of ERK, whereas protein phosphatases are responsible for the rapid dephosphorylation and inactivation of ERK. To clarify whether vimentin can directly affect ERK phosphorylation, we measured the phosphorylation of ERK *in vitro* in the presence of vimentin and alkaline phosphatase (AP) which dephosphorylates and inactivates ERK. Interestingly, we found that vimentin could protect ERK2 from AP-mediated dephosphorylation (II, Fig 3a, and S2C) as previously described in neurons (Perlson, Michaelevski et al. 2006). This suggests that vimentin-expressing cells may enhance ERK signalling by inhibiting ERK-specific phosphatases in cells. As both active ERK and vimentin expression have been linked to cancer cell proliferation and disease progression, we tested how vimentin silencing affected MDA-MB-231 tumour growth on chick embryo chorioallantoic membranes (CAMs). Three days after implantation on CAMs, we detected reduced pERK levels specifically in vimentin-silenced cells in addition to a significant inhibition of tumour growth (II, Fig 2B-C).

The next step was to investigate if ERK could assist in maintaining vimentin levels and further support EMT. Indeed, we observed a significant reduction of vimentin mRNA levels after ERK2 silencing in MDA-MB-231 cells (II, Fig 3D). This was further supported by reduced fluorescence intensity of vimentin networks in ERK2-silenced cells (II, Fig 3E). We next expressed a constitutively active ERK2 construct (MEK_ERK2 fusion, CA_ERK2) in vimentin-negative MCF10A epithelial cells. Strong vimentin staining was observed only in CA_ERK2 expressing cells compared to untransfected cells (II, Fig 3F). Together our findings prove the formation of a reciprocal regulatory complex between vimentin and ERK2.

As mentioned previously, Slug, an important initiator of EMT, displays similar localisation to ERK in TNBC samples. Therefore, we next wanted to investigate if Slug was part of the vimentin-ERK complex involved in EMT regulation. We started by defining the localization of Slug using STED microscopy and subcellular fractionation assays. Slug was mainly found in the nucleus but interestingly a proportion of this transcription factor was localized in the cytosol (13% ± 6%) at vimentin filaments (II, Fig 4A-B). Furthermore, we analysed Slug dynamics with fluorescence recovery after photobleaching (FRAP). FRAP analysis also showed that Slug actively shuttles between the nucleus and

cytoplasm (II, Suppl. Fig S3A-C). The interaction between vimentin and Slug was confirmed with *in situ* PLA (Fig. 4C). Closer investigation with purified recombinant Slug and GST-vimentin showed that vimentin-Slug interaction occurred via direct binding (II, Fig 4D). This was further validated in cosedimentation experiments, in which we detected Slug only in the vimentin-positive insoluble pellet and interestingly, ERK was also present in this same fraction (II, Fig 4E). Moreover, Slug protein levels were reduced in Vim KO MEFs indicating a positive correlation between vimentin and Slug (II, Fig 4F).

Taken together, our results demonstrate that both active ERK and Slug interact with vimentin filaments and there is a direct link between these three important regulators of EMT.

5.2.3 ERK phosphorylates Slug

Since we identified that vimentin interacts with both active ERK and Slug, we next wanted to establish whether ERK and Slug could associate with each other. We studied Slug-ERK interaction in MDA-MB-231 cells overexpressing flag-tagged Slug in response to EGF stimulation to activate ERK. Indeed, we observed co-immunoprecipitation between Slug and active ERK (II, Fig 5A).

As Snail, another member of the Snail transcription factor family, is known to be regulated by post-translational modifications, especially phosphorylation (Zhou, Deng et al. 2004), we next assessed whether Slug could also be subjected to phosphorylation-dependent regulation. We found that Slug is phosphorylated in an ERK-dependent fashion in cells (II, Fig 5B). To elucidate whether ERK-mediated Slug phosphorylation occurs directly, we performed *in vitro* kinase assays with recombinant ERK1 and ERK2 kinases and used active recombinant AKT1 kinase as a control. Both ERK1 and ERK2 were able to phosphorylate Slug *in vitro* (II, Fig 5C). The phosphorylation sites were identified by liquid chromatography-tandem mass spectrometry (LC/MS-MS) analysis (II, Suppl. Fig S4A). Two major serine phosphorylation sites, serine 87 and 104 (S87, S104) were recognised which matched the minimum consensus sequence of the ERK phosphorylation motif (pSP) (II, Suppl. Fig S4B). These consensus sequences were absent in Snail (II, Fig 5D), implying that ERK most likely regulates Slug in a different fashion when compared to Snail. To validate these specific phosphorylation sites in Slug, we used mutagenesis to generate serine to alanine mutations of the sites. Slug S87,104AA mutant protein was used in *in vitro* kinase assays. Results from the assays showed significantly reduced ERK-dependent phosphorylation of this mutant when compared to wt Slug (II, Fig 5E). To study the relevance of ERK-mediated Slug phosphorylation in cells, we ran a Phos-Tag gel from cell lysates overexpressing Slug mutant constructs. We observed a slowly migrating band representing phosphorylated Slug in cells expressing wt Slug and Slug S104A mutant. Importantly, this protein band was absent in cells expressing Slug

S87A and Slug S87,104AA mutants and from the nonphosphorylated recombinant Slug protein used as a control (II, Fig 5F). In conclusion, our data suggest that the S87 residue is the main ERK phosphorylation site in Slug since disrupting the S104 site does not affect the total phosphorylation status of Slug, even though this site is phosphorylated *in vitro*.

To confirm if endogenous Slug is phosphorylated in cells and whether phosphorylation is ERK-dependent, we generated a specific phospho antibody against p-Slug S87. The antibody detected p-Slug from MCF10A and MDA-MB-231 cell lysates but not the nonphosphorylated recombinant Slug protein. The antibody seemed to be specific since the signal was lost upon Slug silencing in MDA-MB-231 cells (II, Fig 5G). Furthermore, antibody specificity was validated in cells expressing Flag-Slug S87A/S87,104AA constructs which exhibited a reduced p-Slug signal compared to wt Slug-expressing cells (II, Suppl. Fig S4D). Next, we treated MCF10A and H-RAS-transformed MCF10A cells with the ERK inhibitor U0126 which clearly reduced the phosphorylation of Slug S87, indicating further that Slug is phosphorylated in an ERK-dependent manner (II, Fig 5G, Suppl. Fig S4C). Importantly, the treatment of MCF10A cells with TGFβ induced the phosphorylation of Slug S87 and this was sensitive to ERK inhibition (II, Fig 5H). In addition, the overexpression of constitutively active CA_ERK2 enhanced the phosphorylation levels of Slug S87 (II, Fig 5I). Taken together, our results show that Slug S87 is phosphorylated in an ERK-dependent manner.

5.2.4 ERK-dependent Slug phosphorylation regulates vimentin and Axl expression

We next investigated if ERK-dependent Slug phosphorylation is important for the regulation of vimentin expression and the EMT phenotype. We used the Slug- and vimentin-negative MCF7 breast cancer cell line as a model and co-transfected these cells with a control Flag or Flag-Slug wt/S87A/S87,104AA constructs together with a vimentin promoter-GFP reporter (Gilles, Polette et al. 1999). The expression of wt Slug strongly induced vimentin transcription, as indicated by the GFP signal, whereas Slug mutants failed to do so (II, Fig 6A). The same was observed with another vimentin reporter construct where the vimentin promoter was fused to luciferase. Vimentin expression was significantly impaired in cells expressing Slug mutants when compared to cells expressing wt Slug. An additional DNA-binding-deficient mutant of Slug (C161,215A;Slug DM) functioned as a negative control and was completely unable to induce vimentin (II, Fig 6B). In addition, in MCF10A cells where the expression of Slug wt was retrovirally transduced vimentin expression was upregulated as detected by Western blotting and immunofluorescence (II, Fig 6C-D). Furthermore, these wt Slug-overexpressing MCF10A cells migrated more when compared to control cells (II, Fig 6E). Since the overexpression of Slug S87A in MCF10A cells also slightly induced migration, it indicates that the

phosphorylation of Slug S87 is at least partially unnecessary for the induction of migration in these cells.

As it is well-characterized that Slug represses E-cadherin expression, we wanted to study if ERK-dependent phosphorylation of Slug is needed for this process. Surprisingly, wt Slug and all the phosphorylation site mutants of Slug were able to inhibit the transcriptional activity of the E-cadherin promoter in E-cadherin reporter assays in both HEK293 and MCF7 cells (II, Suppl. Fig. S5A-B). Moreover, in retrovirally transduced MCF10A cells both wt Slug and the mutants repressed E-cadherin protein levels (II, Suppl. Fig. S5C). In conclusion, our data shows that ERK-dependent phosphorylation has an important role in inducing EMT which is distinct from the previously described phosphorylation-dependent regulation of the Snail family proteins. The ERK-mediated phosphorylation of Slug S87 is essential for the ability of Slug to induce vimentin expression but is dispensable for Slug-mediated E-cadherin repression.

We next investigated whether ERK-mediated Slug phosphorylation could regulate the stability and localization of Slug. The phosphorylation-dependent regulation of these events has been intensively studied, especially in the case of the Snail transcription factor. However, to our surprise, GFP-tagged wt Slug and Slug mutants were all localised predominantly in the nucleus in MCF10A cells (II, Suppl. Fig. S5E). This nuclear localization of both wt Slug and Slug mutants was also observed with Flag-tagged Slug constructs (data not shown) indicating that the nuclear staining was not due to the GFP-tag. In addition, when we silenced vimentin from MDA-MB-231 cells we saw no changes in the abundance of endogenous Slug between the nucleus and the cytoplasm (II, Suppl. Fig. S5F). These results indicate that ERK does not regulate the nuclear localization of Slug and this can be explained by the fact that Slug lacks the phosphorylation-masked nuclear export motif found in Snail (Dominguez, Montserrat-Sentis et al. 2003).

We have previously shown that vimentin regulates EMT induction and breast cancer cell migration by influencing Axl expression (Vuoriluoto, Haugen et al. 2011). To study if ERK-mediated phosphorylation of Slug, which was shown here to be essential for vimentin expression, influences Axl protein levels, we analysed Axl cell surface levels using FACS. We used MCF10A cells in which the expression of Slug wt/S87/S87,104AA was retrovirally transduced. In agreement with the ability of Slug to induce vimentin expression, wt Slug but not S87 or S87,104AA mutants, significantly enhanced Axl cell surface levels (II, Fig 7A). Similar results were also seen when using immunofluorescence staining (II, Fig 7B). Since we know that vimentin, ERK2 and Slug are all needed for MDA-MB-231 cell invasion and that ERK-mediated phosphorylation of Slug S87 is important for Axl induction in MCF10A cells, we investigated if silencing of Axl could inhibit cell invasion. In MDA-MB-231 cells, stable Axl

silencing did not affect vimentin, Slug or pERK protein levels (II, Fig 7C) but significantly impaired the invasion of these cells (II, Fig 7C). These results indicate that Axl contributes to the EMT-related invasion of cells, downstream of EMT-inducing factors. In summary, our data shows that ERK phosphorylates Slug at residue S87 which is needed for Slug-mediated vimentin expression and Axl induction. In addition, the inhibition of Axl or upstream activators of Axl hinders the invasion of MDA-MB-231 cells (Figure 10).

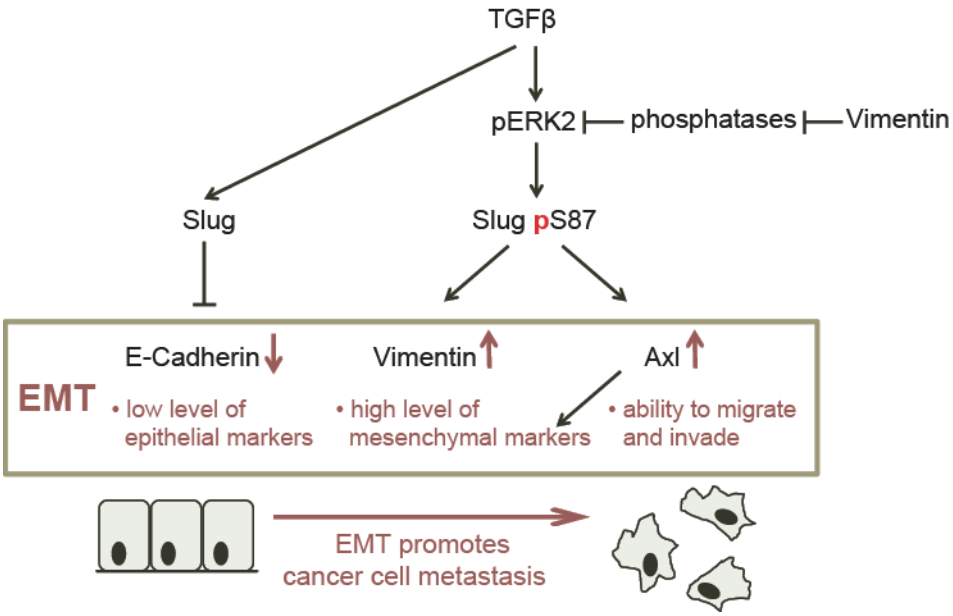

Figure 10. Model for EMT induction by ERK-dependent phosphorylation of Slug. Vimentin and ERK2 interact and form a reciprocal regulatory complex. Vimentin protects ERK2 from dephosphorylation and thus supports ERK activity. ERK2-dependent phosphorylation of Slug at serine 87 is required for induction of vimentin and Axl expression and breast cancer cell invasion. Adapted from (Virtakoivu, Mai et al. 2015).

6. DISCUSSION

6.1 THE REGULATION OF β1-INTEGRIN ACTIVITY BY AKT1 AND AKT2 KINASES (I)

In this thesis work we have demonstrated that AKT kinases regulate β1-integrin activity in prostate cancer cell lines, by showing that inhibition of AKT with a pan-AKT inhibitor induces β1-integrin activation. More precisely, specific silencing of AKT1 or AKT2 isoforms results in increased β1-integrin activity. This could be due to upregulation of β1-integrin activating proteins. However, we did not detect increased levels of either talin or kindlin, the two main inside-out activators of integrins, upon AKT silencing (unpublished data). Integrin activation is often linked to stronger cell-ECM adhesions (Stupack and Cheresh 2002). In line with this, we observed enhanced adhesion to collagen when cells were treated with a pan-AKT inhibitor. In addition, we observed that silencing of AKT1 or AKT2 increased the number and size of vinculin-positive focal adhesions. Altogether, our results illustrate that silencing of AKT1 and AKT2 leads to upregulation of β1-integrin activity and further enhances downstream integrin functions.

It is well-established that integrins can activate downstream signalling such as the PI3K-AKT pathway and in this way drive cell survival and motility (Legate, Wickstrom et al. 2009). In addition, integrins have been shown to regulate the inactivation of AKT kinases by influencing PP2A function (Ivaska, Nissinen et al. 2002). Before our studies, there was no direct implication of AKT1 or AKT2 in regulating β1-integrin activity, at least in prostate cancer. The question that remains is what is the mechanism by which AKT isoforms regulate β1-integrin activity?

There is an increasing body of evidence for negative feedback loops regulating crosstalk between signalling pathways, especially in cancer. For example when activated RTKs induce the AKT pathway, active AKT kinases inhibit FOXO-dependent RTK gene expression creating a negative feedback loop regulating RTKs. In addition, after AKT activation mammalian target of rapamycin complex 1 (mTORC1) and p70 ribosomal protein S6 kinase 1 (S6K1) are activated leading to inhibition of insulin receptor substrate 1 (IRS-1) phosphorylation. Upon AKT inhibition these negative feedback loops are relieved leading to increased RTK gene expression and activity (Chandarlapaty, Sawai et al. 2011, Schwartz, Wongvipat et al. 2015). On the other hand, crosstalk between RTKs and integrins is well-known (Ivaska and Heino 2011). This prompted us to explore what happens to RTK activation upon AKT silencing. AKT1 silencing induced the phosphorylation of several RTKs, such as EGFR and Met compared to control silenced cells. Surprisingly, the phosphorylation of these RTKs was not upregulated in AKT2-silenced PC3

cells. This highlights the importance of understanding the isoform-specific functions of AKT kinases and it will be discussed later on in chapter 6.2 and 6.3. To further prove the connection between AKT1, EGFR/Met and β1-integrin activity, we overexpressed or silenced EGFR and Met either alone or together but we were not able to affect β1-integrin activity with these approaches in these cells (unpublished data). This suggests that AKT1-specific inhibition of β1-integrin activity is a sum of all seven (EGFR, ErbB2, Axl, Met, PDGFR α, c-Ret, VEGFR2) RTKs influenced by AKT1 or that the level of EGFR and Met overexpression was not sufficiently high enough to activate β1-integrin. In addition, PI3K inhibition has been shown to induce ERK signalling in breast cancer (Serra, Scaltriti et al. 2011). This compensatory activation of ERK together with RTK-induced ERK activation could also be involved in the upregulation of β1-integrin activity in AKT1-silenced PC3 cells.

During the course of our studies Iliopoulos and colleagues revealed how AKT isoforms differentially regulate the abundance of miR-200 family members. They showed that in breast epithelial cells activated AKT2 downregulates miR-200 levels. When we studied the levels of miR-200a and miR-200b in AKT1- or AKT2-silenced PC3 cells, we saw that AKT2 silencing induced miR-200a and miR-200b levels whereas AKT1 silencing slightly reduced them. In line with our previous data, we detected increased β1-integrin activity and enlarged focal adhesions after overexpression of miR-200a. Our results illustrate a correlation between AKT2 silencing, upregulation of miR-200 and β1-integrin activation but to unravel the detailed mechanistic regulation behind this would require more investigation. In light of the known role of miR-200 as a suppressor of EMT (Feng, Wang et al. 2014), it might seem a bit unexpected that upregulation of miR-200 leads to activation of β1-integrin. But there are studies showing the opposite roles for miR-200 in cancer progression which are consistent with our results. Dykxhoorn and colleagues showed in mouse breast cancer cell lines that upregulation of miR-200 promotes the dissemination of tumour cells (Dykxhoorn, Wu et al. 2009). In addition, in melanoma cell lines the expression of miR-200 was shown to induce invasion by switching the invasion mode towards a more amoeboid-like state (Elson-Schwab, Lorentzen et al. 2010).

6.2 THE ROLE OF AKT1 AND AKT2 IN PROSTATE CANCER CELL MOTILITY (I)

We have demonstrated that the migration and invasion of AKT1- and AKT2-silenced PC3 cells is increased. This is unsurprising as activation of β1-integrin and enhanced adhesion is closely linked to cell migration and invasion. We noticed that the effect on invasion was much stronger than on migration. The invasion assay that was used in our studies measures the ability of cells to invade through three-dimensional (3D) matrix, in this case matrigel, towards an increasing serum gradient. Matrigel is a basement membrane matrix which

mainly consists of laminin, collagen IV, and different GFs. Therefore, for cells to invade through matrigel they have to degrade and remodel the ECM and this actually closely resembles what occurs during cancer cell invasion in the body. MMPs like MMP-2, MMP-7 and MMP-9 are known to be critical for tumour cell invasion (Stetler-Stevenson, Aznavoorian et al. 1993, Montgomery, Mueller et al. 1994, Hua and Muschel 1996, Sugiura, Shimada et al. 1998). As we saw that the invasive phenotype was β1-integrin-dependent, MMPs are also most likely involved in this process. There are studies showing that active β1-integrin induces the expression of MMPs for example MMP-1 (Riikonen, Westermarck et al. 1995) and MMP-9 (DiPersio, Shao et al. 2000).

We also saw enhanced cell migration in both AKT1- and AKT2-silenced cells but the effect was not as strong as the effect on cell invasion. We studied cell migration using two different methods. AKT2 silencing increased migration significantly when cells were plated on top of CDMs (Even-Ram and Yamada 2005) whereas the increase induced by AKT1-silenced cells was not significant. Surprisingly, when these cells were plated on plastic AKT1 silencing induced a small but significant increase in migration while AKT2 silencing had no effect. The requirements for migration on CDMs more closely resembles those in 3D and even the control cells migrated more on a softer CDM when compared to stiff plastic. There are a few possible explanations why only AKT1 silencing promoted cell migration on plastic. It is well characterised that activated RTKs and their downstream signalling can induce migration also independently of integrins. For example ERBB2 together with EGFR has been shown to induce breast cancer cell migration (Verbeek, Adriaansen-Slot et al. 1998, Marone, Hess et al. 2004). In addition, Axl is an important regulator of EMT and cancer cell migration (Gjerdrum, Tiron et al. 2010, Vuoriluoto, Haugen et al. 2011). Thus, it is possible that AKT1-driven cell migration is due to ERBB2 and EGFR or Axl activation. Rapid formation and turnover of focal adhesions is essential for cell migration (Webb, Parsons et al. 2002). In addition, it has been shown that moderate rather than very high expression of adhesion complexes correlates with motility (Murphy-Ullrich 2001). We observed a strong induction of focal adhesion size and number in AKT1- and AKT2-silenced cells on plastic. Thus, one possibility is that due to this, the turnover of focal adhesions is decelerated and this prevents or decreases cell migration on plastic. Focal adhesion turnover was not investigated in our studies.

6.3 ISOFORM-SPECIFIC FUNCTIONS OF AKT AND ERK KINASES (I),(II)

There is an increasing amount of evidence showing the importance of understanding the isoform-specific functions of kinases in cancer. In this thesis work two separate kinase families, AKT and ERK kinases, were studied. In the case of AKT kinases, several reports demonstrate opposite roles for AKT isoforms in cell migration and invasion (Irie, Pearline et al. 2005, Yoeli-Lerner,

Yiu et al. 2005). According to our studies in prostate cancer both AKT1 and AKT2 function as negative regulators of β1-integrin activity and cell motility through different mechanisms. One could speculate that this was due to a compensatory effect such that inhibition of one AKT isoform would lead to an upregulation of another isoform but this was not the case. The initial findings that AKT1 and AKT2 are negative regulators of β1-integrin activity was seen with several prostate cancer cell lines; using both androgen-sensitive and –insensitive cell lines but the studies of motility were performed only with PC3 cells (androgen-insensitive). In contrast, AKT3 isoform had no effect on β1-integrin activity and cell motility in PC3 cells even though in androgen-sensitive VCAP cells AKT3 was identified to positively regulate β1-integrin activity (Pellinen, Rantala et al. 2012). It has become evident that the regulation of cell invasion by AKT isoforms is strongly cell-type and context-dependent and to obtain a more comprehensive picture of what the role of AKT kinases is in prostate cancer motility, more cell lines should be studied.

In total more than 100 AKT substrates have been reported in the literature (Manning and Cantley 2007) and knowledge about isoform-specific substrates is increasing. For example AKT1-dependent phosphorylation of actin bundling protein palladin facilitates the inhibition of breast cancer migration (Manning and Cantley 2007, Chin and Toker 2010). In contrast, AKT2 has been shown to phosphorylate ezrin which functions as a linker between the actin cytoskeleton and its target proteins (Shiue, Musch et al. 2005). Ezrin has been associated with increased migration (Prag, Parsons et al. 2007, Chen, Gao et al. 2014). This substrate specificity could in part explain the difference between AKT1 and AKT2 in regulation of breast and ovarian cancer motility and possibly also in prostate cancer. In addition, some of the differences in regulating cancer cell motility between AKT isoforms might be due to tissue-specific expression or subcellular localization. Relocation of active AKT kinases from the plasma membrane to different cellular compartments has been reported. AKT1 is mainly localized in the cytoplasm, AKT2 is also found in the cytoplasm but it is located more at the mitochondria whereas AKT3 is located mostly in the nucleus and nuclear membrane. There were no major differences between AKT localisation in breast, prostate and cervical cancer cell lines (Santi and Lee 2010). This suggests that the AKT isoform-related differences observed in distinct cancer types is not due to any altered AKT isoform subcellular localization.

The isoform-specific functions of ERK kinases are not as complicated as with AKT kinases. There are several ERK isoforms but the most commonly expressed ones in human tissues are ERK1 and ERK2. These two isoforms have been considered equivalent until quite recently. Nowadays it is clear that ERK1 and ERK2 isoforms can also have distinct roles in regulating cellular functions. For example transgenic mice lacking ERK1 are viable and suffer only from minor deficiencies whereas ERK2 knockout mice are embryonically

lethal (Pages, Guerin et al. 1999, Mazzucchelli, Vantaggiato et al. 2002, von Thun, Birtwistle et al. 2012). In addition, ERK2 is the dominant isoform regulating cancer cell migration and invasion (Botta, Reginato et al. 2012, von Thun, Birtwistle et al. 2012, Radtke, Milanovic et al. 2013) and this was also confirmed in our studies. It has been shown that ablation of ERK1 results in increased ERK2 signalling (Mazzucchelli, Vantaggiato et al. 2002) and this might be the reason why ERK1 depletion does not have the inhibitory-role for cancer cell motility. In addition, the rate of ERK1 and ERK2 shuttling between the cytoplasm and the nucleus is different. The shuttling of ERK1 is approximately three times slower than that of ERK2 (Marchi, D'Antoni et al. 2008). This could impact on the activation of ERK isoforms and also on the phosphorylation of their target proteins.

6.4 TARGETING AKT AND ERK KINASES IN CANCER (I),(II)

The PI3K/AKT/mTOR and RAS/RAF/MEK/ERK signalling pathways are the most frequently misregulated pathways in cancer. There have been attempts to target AKT kinases in clinical trials. The most developed inhibitor against AKT is the allosteric inhibitor perifosine which prevents the translocation of AKT to the membrane. However, perifosine and other AKT inhibitors have failed to antagonise cancer growth and progression. The most likely explanation for this is that AKT inhibition relieves feedback suppression of IGFR1 and other receptors resulting in the activation of PI3Kα and downstream signalling (Chandarlapaty, Sawai et al. 2011, Schwartz, Wongvipat et al. 2015). Since the existing drugs target all AKT isoforms, the insensitivity could be due to isoform-specific functions. As discussed above, especially in breast cancer, AKT1 and AKT2 have been shown to have opposing roles in regulating tumour growth and invasion. The results gained in this thesis work provide new information about AKT isoform-specific functions in prostate cancer. It also further emphasizes the importance of developing isoform-specific drugs against AKT in the future. In addition, our results suggest that AKT-targeting drugs may not be beneficial for treating patients with androgen-insensitive prostate cancer and instead may promote aggressiveness of the disease.

Combination treatments have emerged as a better way to prolong disease-free or progression-free periods in patients. Since the PI3K/AKT pathway includes a multifaceted network of interactions between different signalling cascades, the pharmacological inhibition of AKT or PI3K may release negative feedback loops as discussed previously. A good example of the benefits of combining treatments is the inhibition of AKT and HER kinase activity. This has been shown to be more effective than the respective monotherapies (Chandarlapaty, Sawai et al. 2011). In addition, one of the compensatory mechanisms following PI3K/AKT inhibition is FOXO-mediated feedback activation of RTKs and downstream activation of ERK kinases (Serra, Scaltriti et al. 2011). One possibility could be to combine PI3K/AKT and MEK inhibitors to overcome the

compensatory activation of the MEK/ERK pathway when inhibiting PI3K/AKT. However, this kind of combination approach might be difficult due to increased levels of toxicity in response to these small molecule kinase inhibitors (Shimizu, Tolcher et al. 2012). At the moment there are several inhibitors against RAF and MEK but the interest of developing ERK1- and ERK2-specific inhibitors has increased. Targeting ERK instead of MEK could decrease the toxicity of combination treatments. Actually, several ERK1/2 inhibitors have entered Phase I clinical trials recently but results from these are not yet available (Samatar and Poulikakos 2014).

Inhibitors against the B-RAF V600E mutant are commonly used in the clinic for the treatment of metastatic melanoma. These drugs improve the progression-free period of many patients but nevertheless lead to eventual drug resistance and relapse. This happens via unknown mechanisms which reactivates the MAPK pathway. Since ERK kinases mediate the activation of both RAS and RAF family of proteins, direct inhibition of ERKs could be a solution to overcome drug resistance (Goetz, Ghandi et al. 2014).

6.5 THE REGULATION OF EMT IN CANCER PROGRESSION (II)

Previously, the concept of EMT during cancer progression has been under debate mainly due to a lack of evidence of EMT in clinical samples. In recent years, several excellent studies have elucidated the prevalence of EMT in cancer. Sarrio and colleagues studied 479 invasive breast carcinomas using immunohistochemical analysis of 28 different molecular markers and found a clustering of EMT markers in the basal-like tumour subtypes (Sarrio, Rodriguez-Pinilla et al. 2008). More recently, Prat and colleagues used microarray analyses to classify a claudin-low subtype of ductal breast carcinoma which also exhibits diminished E-cadherin expression. This analysis revealed that this subtype is enriched with core EMT-inducing transcription factors including Snail, ZEB2 and TWIST1/2 (Prat, Parker et al. 2010). In colorectal cancer, Celesti and colleagues showed that 17 out of 20 colon tumours exhibited a mesenchymal phenotype with TWIST1-positive staining (Celesti, Di Caro et al. 2013). Our findings further confirm occurrence of EMT in clinical tumour samples. We found that in a cohort of 356 breast cancer tissue samples the expression of vimentin and Slug was significantly higher in triple-negative samples. In addition, we saw that the level of Slug and active ERK was stronger at the tumour periphery.

Even though many mesenchymal markers and EMT inducers have been identified and linked to cancer progression, the mechanism regulating their expression and activity is still often unclear. In this thesis work we shed light on this complicated regulation.

6.5.1 The role of vimentin in EMT

Previous studies by other and work from our laboratory identified vimentin as an active contributor and signalling hub during EMT (Mendez, Kojima et al. 2010, Vuoriluoto, Haugen et al. 2011). The detailed knowledge on how vimentin regulates EMT is still unclear. Results presented in this thesis work shed light on this complicated regulation. We see a strong correlation between high vimentin expression and activation of ERK and this was consistent between different cell types (MDA-MB-231 cells, TGFβ treated MCF10As and MEFs). This suggests that in vimentin-expressing mesenchymal cells vimentin could maintain ERK activation as has been shown to occur in neurons (Perlson, Michaelevski et al. 2006). Actually, we observed that *in vitro* with pure recombinant components, vimentin protects ERK2 from AP-mediated dephosphorylation. We propose that in cells vimentin could antagonise phosphatases that dephosphorylate and inactivate ERK2. Our results demonstrate that vimentin functions as an essential signalling scaffold by supporting ERK2 activity, In addition we saw that ERK can regulate vimentin expression. Our data defines the formation of a reciprocal vimentin-ERK regulatory complex at the onset of EMT. TGFβ and RAS signalling are important activators of EMT and the activation of both of these pathways leads to ERK activation. Subsequently, ERK regulates vimentin, a known marker of EMT. TGFβ and RAS signalling are known to induce PI3K-AKT activation as well and AKT1 was shown to directly phosphorylate vimentin at S39 residue. This induces migration and invasion and enhances tumour growth and metastasis *in vivo* (Zhu, Rosenblatt et al. 2011).

As we detected the presence of both Slug and ERK at vimentin filaments and as these proteins cosedimented together, we wanted to further study the relationship between ERK and Slug. We were able to confirm an interaction between Slug and active ERK in cells and observed ERK-dependent phosphorylation of Slug. LC/MS-MS analysis, revealed serine 87 and serine 104 in Slug as specific ERK phosphorylation sites. We concentrated our studies on phosphorylation of the S87 site. Together our results suggest that vimentin acts as a bridge between active ERK and Slug and that ERK-mediated phosphorylation of Slug potentially occurs on vimentin filaments. To prove this, ERK-mediated phosphorylation of Slug should be studied in vimentin knock-out MEFs or vimentin-silenced cells. By live-cell imaging we observed that Slug actively shuttles between the nucleus and the cytoplasm. ERK isoforms have also been shown to shuttle between the cytoplasm and the nucleus with ERK2 demonstrating more rapid dynamics (Marchi, D'Antoni et al. 2008). One possibility could be that vimentin filaments only facilitate the ERK-Slug interaction but the actual phosphorylation occurs in the nucleus. ERK and Slug could even be potentially transported together to the nucleus.

6.5.2 The role of Slug transcription factor in EMT

The best-characterized function of Slug in EMT induction is its role in suppression of E-cadherin. Interestingly our results demonstrate that ERK2-dependent phosphorylation of Slug S87 is dispensable for Slug´s ability to repress E-cadherin but instead is involved in induction of vimentin expression in the early onset of EMT. The binding of Slug to the E-cadherin promoter occurs via C-terminal zing-finger domains (Nieto 2002). For the proper repression of E-cadherin the SNAG domain (amino acids 1-9 of slug) is required. Additionally, it has been proposed that the deletion of the SLUG domain (amino acids 96-123) also impairs the repression ability of Slug by approximately 20 percent whereas SNAG-deletion completely abolishes the suppression of E-cadherin (Molina-Ortiz, Villarejo et al. 2012). The fact that the Slug S87 phosphorylation site identified by us is located outside of the SLUG domain is fully in-line with the dispensable role of this phosphorylation event in E-cadherin suppression. Snail belongs to the same transcription factor family as Slug, and the phosphorylation of Snail has been investigated more extensively. ERK2 has been shown to phosphorylate Snail and this induces EMT by regulating the nuclear accumulation of Snail rather than its direct binding to the E-cadherin promoter. We saw that ERK-dependent phosphorylation of Slug does not influence the cellular localization of Slug or its stability. Slug wt and S87 mutant were mainly localized to the nucleus, thus from this point of view it is not surprising that both of these can supress E-cadherin. The molecular mechanism regulating Slug nuclear export is not understood, even though GSK3β-mediated phosphorylation of Slug has been proposed to induce cytoplasmic localization of Slug (Kim, Kim et al. 2012). By contrast, the mechanism of Snail nuclear export is better characterized. Snail, but not Slug, contains a nuclear export signal (NES) which is important in its cycling between the nucleus and the cytoplasm. Next to this NES sequence there is a serine-rich domain (SRD). The phosphorylation of SRD has been shown to hinder the interaction between the SRD and zinc finger domains priming the NES domain for CRM1 transporter binding and initiation of nuclear export (Dominguez, Montserrat-Sentis et al. 2003). Despite this reported mechanism, similar to our results with Slug, ERK2-mediated phosphorylation of Snail does not drive Snail cytoplasmic localization.

As mentioned before, Slug post-translational modifications have not been studied extensively. Most likely there are additional phosphorylation sites in Slug, modified by other kinases which further regulate Slug´s ability to supress E-cadherin expression. In the case of Snail, phosphorylation of S11 and S92 is needed for E-cadherin suppression. Protein kinase A (PKA) was suggested to phosphorylate S11 and casein kinase-2 (CK2) to phosphorylate S92 (MacPherson, Molina et al. 2010). Phosphorylation of Slug by these kinases has never been studied. Additionally, the binding of corepressors has been reported to be essential for the repressive function of transcription factors. The

SNAG domain of Slug has been shown to be important for recruitment of nuclear receptor corepressor (NCoR) corepressor and SNAIL domain for the recruitment of the C-terminal binding protein 1 (CtBP1) corepressor (Molina-Ortiz, Villarejo et al. 2012). As Slug S87 is not within these domains, its phosphorylation most likely does not affect the recruitment of these particular corepressors.

We show that ERK-mediated phosphorylation of Slug S87 is required for induction of vimentin expression. How this occurs is still unresolved. The vimentin promoter lacks consensus Slug-binding sites (the E-box) suggesting that Slug would not bind vimentin promoter directly. Additionally our *in vitro* binding assays with purified Slug and vimentin promoter indicates that there is no direct interaction as such. One possible mechanism could involve recruitment of coactivators. With assistance from other proteins, Snail has been shown to bind fibronectin promoter directly even though this promoter also lacks a consensus binding site for Snail and Slug (Stanisavljevic, Porta-de-la-Riva et al. 2011).

Despite the fact that stable expression of Slug S87A mutant was unable to trigger vimentin expression in the early onset of EMT, we noticed that after several passages the mutant Slug was capable of inducing a complete EMT phenotype (unpublished data). Most likely this is due to the ability of Slug S87A to suppress the expression of E-cadherin. The repression of E-cadherin could result in a “cadherin switch” where the expression of neuronal cadherin (N-cadherin) increases to balance the downregulation of E-cadherin (Wheelock, Shintani et al. 2008). The other possibility is that E-cadherin repression could lead to β-catenin translocation to the nucleus where it enhances Wnt signalling and leads to EMT. Additionally, related to a complete EMT switch in phenotype, vimentin levels were upregulated even in Slug S87A-expressing cells. This suggests that once the epithelial cells have accumulated enough mesenchymal cell properties, the regulatory mechanisms that were effective in the early initiation of EMT are not sufficient anymore. Indeed, this seems to be the case with Slug. One hypothesis could be that other EMT mediators like Snail could induce vimentin expression independently of Slug after E-cadherin repression.

6.6 TARGETING EMT IN CANCER THERAPY (II)

The EMT process has been linked to all steps of metastasis. It has been shown by various excellent studies that disseminated tumour cells (DTC) are enriched with EMT properties, especially the vimentin expression (Derksen, Liu et al. 2006, Huang, Yang et al. 2009, Ota, Li et al. 2009, Stoletov, Kato et al. 2010, Bonnomet, Syne et al. 2012, Shibue, Brooks et al. 2012). Interestingly, in recent years the EMT process has been linked to stem cells. Cells that have undergone EMT have been shown to gain several properties associated with

the evolving concept of cancer stem cells (CSC), a highly tumorigenic subpopulation of cells within a tumour (Mani, Guo et al. 2008, Morel, Lievre et al. 2008). Thus, it would make perfect sense to target EMT for cancer treatment and several ideas have been proposed. For example high-content drug screens have been performed to find possible drugs against EMT in response to several EMT-inducing signals. Many compounds were identified including rapamycin against TGFβ-induced EMT and inhibitors against MEK in response to HGF, EGF and IGF-1 (Reka, Kuick et al. 2011, Chua, Sim et al. 2012). Targeting EMT is challenging due to both the strong link between EMT and development of drug resistance and also the lack of proper targets in the central EMT program.

What is the significance of this thesis study? Our results bring new mechanistic information of how EMT is initiated. In addition, these data reveal a single phosphorylation site within Slug that is capable of determining the ability of this transcription factor to induce EMT through vimentin and Axl expression and provides a functional distinction between Slug-mediated gene expression and Slug-dependent gene suppression. Most importantly this work brings further information about the functional role of vimentin during EMT. Our work and studies done by others highlight the essential role of vimentin in cancer cell migration and invasion and maintaining the signalling needed for these processes. Since the main EMT transcription factors have been technically challenging to target, maybe targeting vimentin could solve this problem. In fact, a compound called Withaferin A has been shown to target vimentin in STS (Lahat, Zhu et al. 2010). In a panel of STS xenograft experiments WFA was shown to inhibit STS growth, local recurrence and metastasis. But since WFA has been suggested to target other proteins like AKT, FOXO-3 and PAR-3 (Oh, Lee et al. 2008, Stan, Hahm et al. 2008), it would be useful to generate more specific compounds that target vimentin only. Since vimentin knockout mice are viable and fertile, targeting vimentin would be safe and would not induce severe side effects. In addition to anti-vimentin drug development, vimentin could be used as a biomarker to predict the re-occurrence of the disease and in that sense also help to plan treatment among cancer patients.

Nevertheless, one drawback in targeting EMT and DTCs could be the occurrence of MET during colonization. This hypothesis was proposed in 2002 by Jean Paul Thiery who suggested that DTCs have to regain epithelial properties and lose mesenchymal characteristics to be able to form macrometastasis in distant organs (Thiery 2002). In line with this hypothesis, reports have shown that EMT induction by Snail or ZEB2 suppresses cell growth by inhibiting the activity of cyclin D (Vega, Morales et al. 2004, Mejlvang, Kriajevska et al. 2007). As one of the requirements for colonization is induced proliferation, the MET process may be essential for providing a growth advantage. In addition, a recent report demonstrated that downregulation of Prrx1, a newly discovered EMT inducer, was essential for lung metastasis after

tail vein injection of breast cancer cells (Ocana, Corcoles et al. 2012). Would it then be beneficial to target mesenchymal cells after all? In my opinion it is a manner of timing. To target EMT in the early stages of tumour development and at the beginning of the metastatic cascade could be the most beneficial option. In general the future of cancer therapies should focus on the development and testing of combination treatments. One possibility would be to combine existing treatments with EMT inhibitors to sensitise tumour cells to chemotherapy.

7. CONCLUSIONS

Activation of the PI3K/AKT pathway is implicated in many cancer types and inhibition of PI3K or its downstream components, including AKTs, are considered as attractive drug targets. However, several studies have highlighted the complexity of biological outcomes obtained upon AKT inhibition including the potential cell type-specific effects of AKT isoforms on cell migration and invasion. This emphasises the importance of studying AKT isoforms separately. Our results demonstrate that, in contrast to breast cancer, in prostate cancer, both AKT1 and AKT2 function as negative regulators of cell migration and invasion. We show that downregulation of AKT1 and AKT2, but not AKT3, induced activation of cell surface β1-integrins and enhanced cell adhesion, migration and invasion. Since it is known that β1 integrin is consistently upregulated in prostate cancer, our findings could at least partially explain this upregulation. In summary, our results illuminate the isoform-specific functions of AKT kinases in prostate cancer.

In breast cancer, EMT characteristics are enriched in the aggressive and metastatic TNBC subtype, suggesting a role for EMT in breast cancer metastasis. Our results describe the formation of a novel vimentin-ERK complex which mediates the regulation of Slug transcriptional activity. We observed overlapping localization of vimentin, ERK and Slug in clinical specimens of TNBC and depletion of any of these three proteins inhibited breast cancer cell invasion. Our findings identify a direct interaction between vimentin and ERK which facilitates ERK-mediated phosphorylation of Slug at serine 87. This ERK-dependent phosphorylation of Slug is required for Slug's ability to induce expression of vimentin and Axl, two key regulators of EMT, and is not required for repression of E-cadherin. Taken together, our results define a prerequisite for ERK-mediated phosphorylation of Slug in EMT induction and regulation of breast cancer cell motility.

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