DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH
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SEARCH FOR MARKERS AND MOLECULAR MECHANISMS OF AGGRESSIVE ENDOMETRIAL CANCER: PROFILING OF AGGRESSIVE VS NON-AGGRESSIVE ENDOMETRIAL CANCERS

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Education is the most powerful weapon which you can use to change the world."

Nelson Mandela

To my beloved and ever supportive fiancé and family

ABSTRACT

Endometrial cancer is the seventh frequent type of cancer among women. Identification of new prognostic markers is important to optimize treatment and follow-up of all EC patients. Understanding the molecular mechanisms of carcinogenesis may pave through discovery of further EC molecular markers.

Cohort studies often disregard the individual features of tumors. Since such features may represent an opportunity to individualize cancer treatment, an innovative approach for their assessment should be developed. In this study (paper I), we addresses the previously overlooked individual characteristics of endometrial cancers, which could serve as a wellspring of information regarding specific molecular processes; regulators of such processes could potentially be useful for predicting aggression in individual endometrial tumors. Systemic analysis of individual proteome profiles represented that different proteins may be impacted in the individual endometrial tumors of different patients, but the impact of these proteins on basic cell functions may still be similar. The correlation between publically available gene expression data sets of profiling of endometrial tumors and our proteome profiling supports the conclusion that individual tumor features are doubtlessly crucial in endometrial tumorigenesis and are not inconsistent individual variations. IHC validation using tissue microarray analysis of MST1 and PKN1 proteins suggested their potential to serve as predictive biomarkers for endometrial cancer as well as efficacy of this approach.

Transforming growth factor-β (TGFβ) and epidermal growth factor (EGF) are two potent regulators of tumorigenesis. Signaling cross-talk between TGFβ and EGF involves a number of regulators which define the impact on cell physiology (Paper II and III). In paper II, we discuss mammalian sterile-like 1 kinase (MST1) as a negative regulator of combined TGFβ and EGF signaling. We observed that enhanced expression of MST1 inhibited the combined action of TGFβ1 and EGF on cell invasiveness, migration and proliferation. Monitoring of the intracellular regulatory proteins showed that the MST1 contribution to TGFβ-EGF cross-talk may involve focal adhesion kinase and E-cadherin, but not activation of Smad2. Our data elucidated the negative feedback role of MST1 on TGFβ1-and EGF-regulated cell invasiveness, migration and proliferation.

Our results from paper III demonstrated that protein kinase N1 (PKN1) modulated responses of HEC-A-1 endometrial cancer cells to TGFβ1 and EGF. PKN1 had an inhibitory effect on stimulation of cell migration, and PKN1 kinase activity was required for the inhibitory effect of TGFβ and EGF on cell proliferation and invasiveness. We observed that phosphorylation of Smad2, FAK and Erk1/2 correlated with cellular response to TGFβ1 and EGF. PKN1 modulates TGFβ and EGF-dependent regulation of cell proliferation, migration and invasiveness, and is therefore a component of the signaling network downstream of TGFβ and EGF.

Thus, our findings provided insights into different mechanisms of tumorigenesis and on the impact of cross-talk between signaling pathways on tumor development.

LIST OF PUBLICATIONS

I.

Attarha S, Andersson S, Mints M, Souchelnytskyi S*. **Individualised proteome profiling of human endometrial tumours improves detection of new prognostic markers**. Br J Cancer. 2013 Aug 6;109(3):704-13.

II.

Attarha S, Andersson S, Mints M*, Souchelnytskyi S*. **Mammalian sterile-like 1 kinase inhibits TGFβ and EGF⁻ dependent regulation of invasiveness, migration and proliferation of HEC-1-A endometrial cancer cells.** Int J Oncol. 2014 Aug;45(2):853-60.

III.

Attarha S, Kanth R, Andersson S, Mints M*, Souchelnytskyi S*. **PKN1 modulates TGF-β and EGF signaling in HEC-1-A endometrial cancer cell line**. Onco Targets Ther. 2014 Aug 4;7:1397-408.

ADDITIONAL PUBLICATIONS

IV.

Kanth R, **Attarha S**, Santos CM, Kolakowska J, Funa K*, Souchelnytskyi S*. **Proteomics of dedifferentiation of SK-N-BE2 neuroblastoma cells**. (Under revision Cancer Science)

V. Santos C, **Attarha S**, Kanth R, Costa J, Barral-Netto M, Brodskyn CI*, Souchelnytskyi S*. **Proteome profiling of Human Cutaneous Leishmaniasis lesion**. (Accepted to J Invest Dermatol.)

VI. Nikoshkov A, Broliden K, **Attarha S**, Sviatoha V, Hellström A.C, Mints M, Andersson S*. **Expression pattern of the PRDX2, RAB1A, RAB1B, RAB5A and RAB25 genes in normal and cancer cervical tissues.** (Accepted to Int J Oncol)

VII. **Attarha S***, Mints M, Andersson S, Souchelnytskyi S. **Endometrial cancer and application of proteomics**. Exp Oncol. 2011 Sep;33(3):174-7

CONTENTS

LIST OF ABBREVIATIONS

2DE	2 dimensional gel electrophoresis
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AKT	Protein Kinase B
ALK	activin receptor-like kinase
BSA	Bovine serum albumin
Cdc42	cell division cycle 42
CDKN2A	cyclin-dependent kinase inhibitor 2A
CT	Computed tomography
DTT	dithiothreitol
EC	Endometrial Cancer
ECM	extracellular matrix
EGF	epidermal growth factor
EMT	epithelial to mesenchymal transition
ERBB2	erythroblastic leukemia viral oncogene homolog 2
ERK	extracellular signal-regulated protein kinases
FBS	fetal bovine serum
FAK	Focal adhesion kinase
FGFR2	fibroblast growth factor receptor 2
FOXO1	forkhead box O1
FOXO3	forkhead box O3
GO	gene ontology
GTPase	guanosine triphosphate hydrolase
IEF	isoelectric focusing
IHC	immunohistochemistry
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
LAP	latency-associated peptide
LC	liquid chromatography
LTBP	latent TGFβ-binding protein
L-TGF-β	latent-transforming growth factor beta
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight
MAPK	mitogen-activated protein kinase
MMPs	matrix metallopeptidases
MRI	Magnetic resonance imaging
MS	mass spectrometry
MST1	Mammalian sterile-like 1 kinase
mTOR	Mammalian target of rapamycin
NF-kB	nuclear factor kappa B
PAK1	p21 protein-activated kinase 1
Para6	ParA domain protein
PBS	Phosphate-buffered saline

PDK1	pyruvate dehydrogenase kinase
PET	Positron emission tomography
pI	isoelectric point
PI3K	phosphatidylinositol 3-OH kinase
PKA	Protein kinase A
PKN1	protein kinase N1
POD	peroxidase
PTEN	phosphatase and tensin homolog deleted from chromosome 10
PTM	post-translational modification
RAC1	ras-related C3 botulinum toxin substrate 1
Ras	retrovirus-associated DNA sequences
Rb	Retinoblastoma
RhoA	ras homolog family member A
R-Smad	activin receptor-like kinase
S100A4	S100 calcium binding protein A4
SAPK	stress-activated protein kinase
SDS	Sodium dodecyl sulfate
ShcA	type III chaperone protein ShcA
Smad	SMA-related, mothers against decapentaplegic protein
SNP	Single-nucleotide polymorphism
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor-β receptor
TP53	tumor protein p53
TMA	tissue microarray

1 Introduction

1.1 ENDOMETRIAL CANCER

Endometrial cancer (EC) is the seventh frequent type of cancer among women and the most common malignancy of the female reproductive system, accounting for 6% of all female cancers in industrialized countries and carries a 2-3% lifetime risk for acquiring the disease (Salvesen *et al*, 2012)(Engelsen *et al*, 2009)(Attarha *et al*, 2011).The incidence of endometrial cancer is estimated at 15-20 per 100,000 women per year, which translates to 81,500 new cases each year (Martinho *et al*, 2012)(Dedes *et al*, 2011). The exact etiology of endometrial cancer remains unknown. The disease is uncommon before age 40, with <20% occurring before menopause (Engelsen *et al*, 2009).

1.1.1 Types of Endometrial cancer

Most endometrial cancers (90%) are adenocarcinomas that arise from uterine epithelial cells (Kim *et al*, 2010). All endometrial carcinomas are categorized into two groups depending on histopathology, each displaying a clear association with molecular findings (Muinelo-Romay *et al*, 2011). Low-grade, early-stage, highly differentiated estrogen-related endometrioid adenocarcinomas (Type I) account for more than 80% of cases, and usually have a good prognosis. Endometrial hyperplasia in the setting of excess oestrogen exposure gives rise to endometrioid adenocarcinoma, which usually affects pre- and perimenopausal women (Mhawech-Fauceglia *et al*, 2012)(Engelsen *et al*, 2008)(Kim *et al*, 2010)(Martinho *et al*, 2012). Non-endometrioid (Type II) tumors (papillary serous and clear cell tumors) are not estrogen-related and show a 3–10% variable prevalence among all endometrial carcinomas; typically, these are high-grade tumors that are poorly differentiated and highly invasive in the myometrium, showing a more aggressive clinical course (Mhawech-Fauceglia *et al*, 2012)(Engelsen *et al*, 2008)(Martinho *et al*, 2012). These tumors usually affect older postmenopausal women with atrophic non-neoplastic endometrium (Kim *et al*, 2010)(Attarha *et al*, 2011).

One third of all cases who die from this disease were initially diagnosed with early-stage disease (Trovik *et al*, 2011). Early appearance of symptoms allows diagnosis at an early stage when the disease is still restricted to the uterus, and is associated with a high rate of survival (Dizon, 2010). However, a subset of endometrial tumors which display aggressive behavior characterized by high histological grade, as well as by lymphovascular and myometrial invasion, is associated with poor prognosis. In about 25% of cases, surgical staging revealed extrauterine disease (Muinelo-Romay *et al*, 2011).

Invasion and metastatic spread are necessary for cancer progression. Metastatic disease represents an advanced stage of most cancers (Mannelqvist *et al*, 2011). The primary sites affected by early development of endometrial cancer include the adnexa and pelvic viscera, as well as the pelvic and paraaortic lymph nodes; a low incidence of distant metastases occurs through hematogenous spread. The molecular mechanisms underlying aggressive transformation and dissemination are largely

unknown (Muinelo-Romay *et al*, 2011). Patients with aggressive disease who are at risk of recurrence receive adjuvant treatment after surgery. Although these patients initially show a good response to localized tumor surgery, they often succumb to metastatic disease associated with a reduction in overall survival (Abal *et al*, 2007).

1.1.2 Endometrial Cancer Detection and Treatment

Despite the growing body of knowledge that points to a unique distinction between type I and type II endometrial cancers, the current basis for management of EC involves various surgical procedures (complete hysterectomy, bilateral salpingo-oophorectomy) and surgical staging. Patients with more aggressive disease who are at high risk of recurrence are treated with additional surgical intervention including omentectomy, as well as para-aortic and pelvic lymphadenectomy. Adjuvant therapy is necessary in this group of patients. Regardless of the type of endometrial carcinoma, postoperative treatment includes radiation therapy and chemotherapy regimens that provide similar benefit in both types (Dedes *et al*, 2011)(Trovik *et al*, 2012)(Lambropoulou *et al*, 2010)(Frederick & Straughn, 2009). Of all endometrial cancers, many occur in older women with co-morbidity, obesity and a high risk of complications in response to aggressive treatment (Dizon, 2010).

Therefore the ultimate goal is to find the best treatment for women with endometrial cancer, while avoiding overtreatment or undertreatment. However, the risk associated with individual patients can usually be determined postoperatively. Due to limitations imposed by pathological factors preoperatively or intraoperatively, selection of patients appropriate for lymphadenectomy is difficult. Preoperative histological assessment of endometrial biopsy often differs from the final pathology reading (Goudge *et al*, 2004). Imaging procedures such as MRI, CT and PET are limited in terms of both specificity and sensitivity in their ability to define the extent of myometrial invasion and lymph node metastasis, as well as cervical and parametrial involvement.

Specific therapies targeting each type of endometrial cancer have not yet been introduced. Thus there is a need to individualize type-specific therapy in order to avoid unnecessary surgical and adjuvant treatment and their associated side effects (Engelsen *et al*, 2009). Identification of new prognostic markers is important to optimize treatment and follow-up of all EC patients (Mannelqvist *et al*, 2009).

1.1.3 Endometrial Tumorigenesis

1.1.3.1 Molecular mechanism of endometrial tumorigenesis

The molecular mechanism underlying endometrial carcinoma has not yet been fully explored. Differences in molecular alterations distinguish the types of endometrial carcinomas. Type I tumors are diploid, microsatellite-unstable, frequently hormone-receptor positive and demonstrate dysregulation of the PI3K/PTEN/AKT molecular pathway with loss of PTEN gene functionality, oncogenic mutations, and overexpression of upstream tyrosine kinase growth factor receptors, causing uncontrolled cell survival and proliferation. Type II tumors are often aneuploid, more common among older women and demonstrate alterations in CDKN2A, TP53, and

ERBB2 (Salvesen *et al*, 2012), as well as loss of E-cadherin expression, and overexpression of HER2 (Figure 1)(Dedes *et al*, 2011).
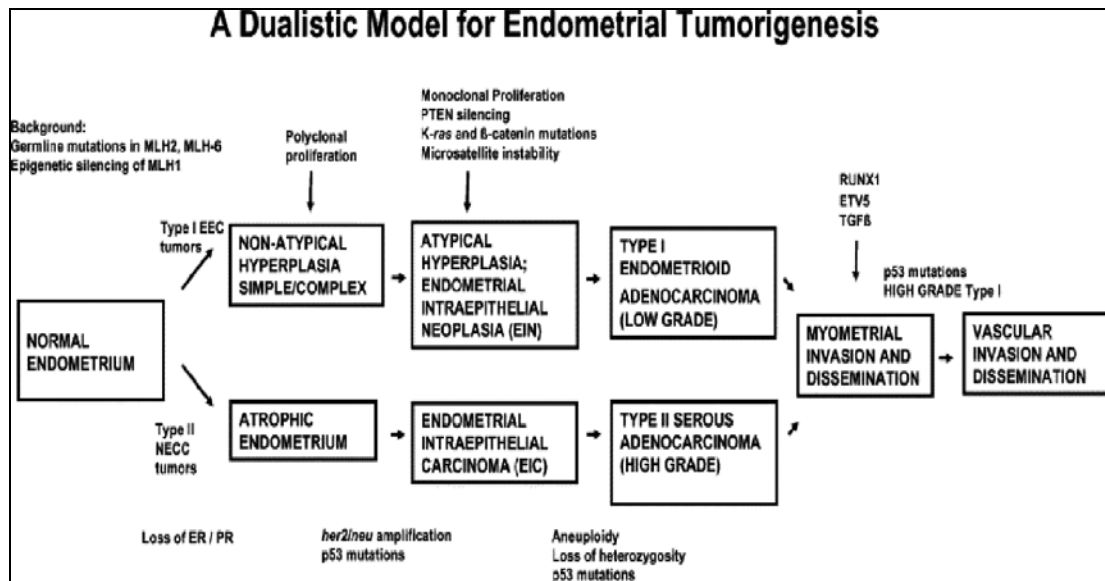

Figure 1. *A model for progression of endometrial carcinoma associated with a higher number of chromosomal abnormalities in endometrioid lesions than in hyperplasia, presence of genetic alterations in atypical hyperplasia and elevated levels of these in well-differentiated carcinomas compared with atypical hyperplasia. Reprinted from Doll et al., Journal of Steroid Biochemistry & Molecular Biology 2008, with permission from Elsevier (Doll et al, 2008).*

1.1.3.2   In vitro models of endometrial cancer

Most in vitro models pertaining to human endometrial epithelial cells were developed by injecting fragments of tumor or specific numbers of cells into fat pads of isogenic animals, specifically athymic nude mice or rats (Vollmer, 2003).

Two factors affecting the invasive properties of endometrial cancer cells in vitro are cell lines origin and level of differentiation. Among the most commonly studied cell lines, SNG-M (metastatic lymph node derived cell line) and NUE-1 (poorly-differentiated cancer cell line) are classified to be more invasive compared to HEC-1BE and HEC-1A (moderately-differentiated) or HEC-6 and Ishikawa (well-differentiated) cell lines (Mori *et al*, 1994).

The first endometrial cancer cell line, HEC-1 (human endometrial cancer-1), was generated in May 16, 1968 by culturing uterus tissue extract of a woman with grade 2 endometrial cancer. Initially, for selection of epithelial cell type, a plasma clot culture was created; expanded cells were cultured over 3 generations and subsequently placed into monolayer culture. The HEC-1-A monolayer cell culture demonstrated steady, continuous logarithmic growth with 2 terminated cases of normal endometrial cells between 100 and 150 days. HEC-1 cell population doubling time was estimated at 31 hours, with 12 hours of G1 phase, 6 hours G2 and 13 hours of S (Kurarmoto *et al*, 2002).

Many proteins were shown to have a strong impact on the invasiveness of endometrial cancer. Non-invasive cell lines transfected with such regulators can also serve as an in vitro model for invasiveness. One example is the S100A4 model, in which endometrial cancer cell lines are transfected with S100A4, which mediates cancer invasion and serves as a target of TGF-β1 signaling (Xie *et al*, 2009).

Further effort is required to explore the underlying mechanism of endometrial cancer invasiveness and aggressiveness, which could provide greater insight of both diagnostic and therapeutic importance.

1.1.3.3 Molecular mechanism of cancer invasion

Endometrial cancer invasion is a complex biological process involving a series of cellular events in which E-cadherin down-regulation is a key component of the EMT process, along with alterations of other cell-cell contact molecules that affect the migratory properties of cells. Moreover, other modified transcription factors and signaling pathways affect the ability of cancer cells to invade the myometrium, and are involved in changes in histological grade and metastatic potential (Figure 2)(Abal *et al*, 2007).
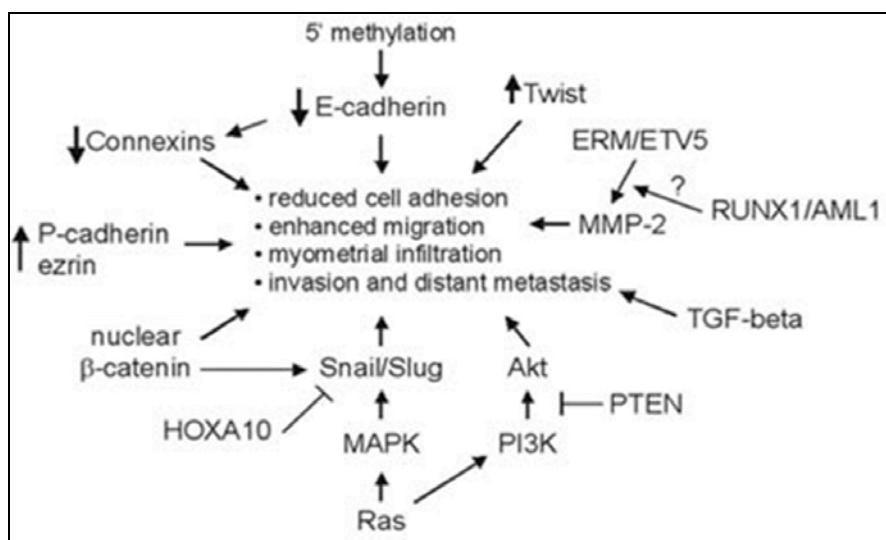

Figure 2. Crucial molecular events in progression of endometrial cancer invasion. Reprinted from Abal et al., Clinical and Translational Oncology 2007, with permission from Springer (Abal et al, 2007).

The invasive nature of cancer is a complicated biological process which involves modifications in cell motility and adhesion that enables tumor cells to bind to and migrate through the extracellular matrix (ECM) and to invade the basal layer of connective tissue (Oh *et al*, 2009). Some of these alterations develop at cell/ECM contact points known as focal adhesions, which consist of membrane-associated, and cytoskeletal components, ECM proteins, as well as intracellular signaling molecules. One of these putative signaling molecules is a protein tyrosine kinase called FAK (Focal adhesion kinase)(Owens *et al*, 1995).

Several factors, including G protein-coupled receptors, integrin receptors and Src activate FAK. Integrins are transmembrane receptors that transmit growth factor signals by means of contact with the extracellular matrix. Autophosphorylation of tyrosine Y397 FAK is a key regulator of integrin signals in cancer cells. Overexpression of FAK has been investigated in several human cancers, including colorectal, head and neck (HNSCC), breast, sarcoma, prostate, and thyroid, and correlates with survival outcomes (Thanapprapasr, 2011)(McLean *et al*, 2005). The role of FAK kinases has been widely studied in cell survival (Tamura *et al*, 1999), migration (Turecková *et al*, 2009), invasion (Siesser & Hanks, 2006) and tumor angiogenesis (Haskell *et al*, 2003)(Figure 3).
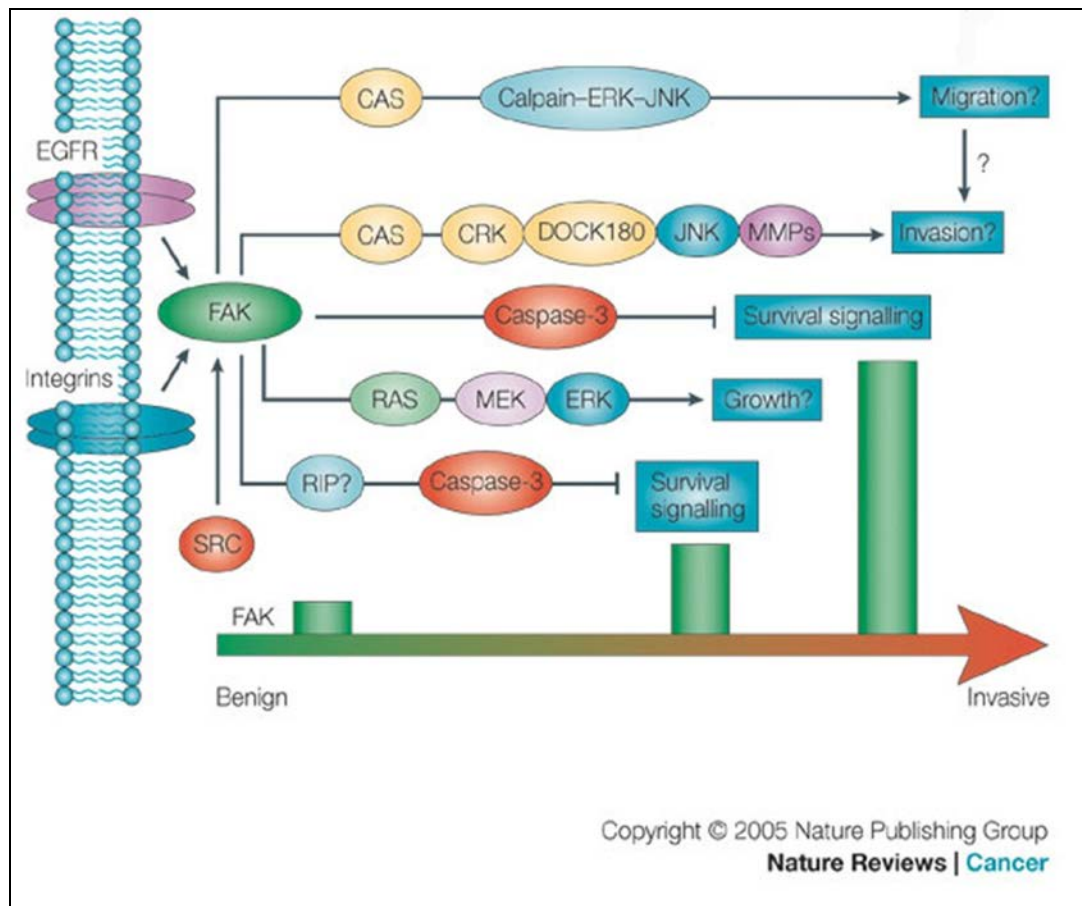

Figure 3. Schematic representation of how FAK is involved in cancer progression. FAK induces the invasive pathway by downstream signalling to regulators such as JUN N-terminal kinase (JNK) and RAC1, as well as to matrix metalloproteinases (MMPs). FAK acts as a key downstream effector of growth-factor-receptor and integrin signalling inhibiting apoptosis and taking part in cell growth via the RAS–MAPK (mitogen-activated protein kinase) pathway. Reprinted from McLean et al., Nature Reviews Cancer 2005, with permission from Nature Publishing Group (McLean et al, 2005).

Epithelial-derived cancers can progress to an invasive, metastatic state, which correlates with a change from an adherent, epithelial nature into a motile, fibroblast-like morphology, an epithelial to mesenchymal transition (EMT)(Peinado *et al*, 2007). Mesenchymal cells are defined by three major characteristics in their cellular

phenotype and their behavior (Figure 4): (1) loss of strong adhesive epithelial cell–cell contacts and (2) the acquisition of a spread, spindle-shaped morphology with markers from cell–cell junction proteins, i.e., E-cadherin and cytokeratin intermediate filaments (specific to epithelial cells) to vimentin filaments and fibronectin, and (3) increased motility that facilitates invasion through the extracellular matrix. These changes are not necessarily observable during the EMT process, although single cell acquisition of the ability to migrate and invade the extracellular matrixe is considered a functional hallmark of EMT (Colas *et al*, 2012).

Further research is required to elucidate the details surrounding the mechanism of cancer invasion and key regulators of this process in order to develop drug targets to block invasive-specific pathways. Since proteins are typically the targets for diagnosis and treatment of cancer, it is anticipated that proteomics analysis of such proteins will provide a comprehensive description of all proteins involved in the invasive mechanism.
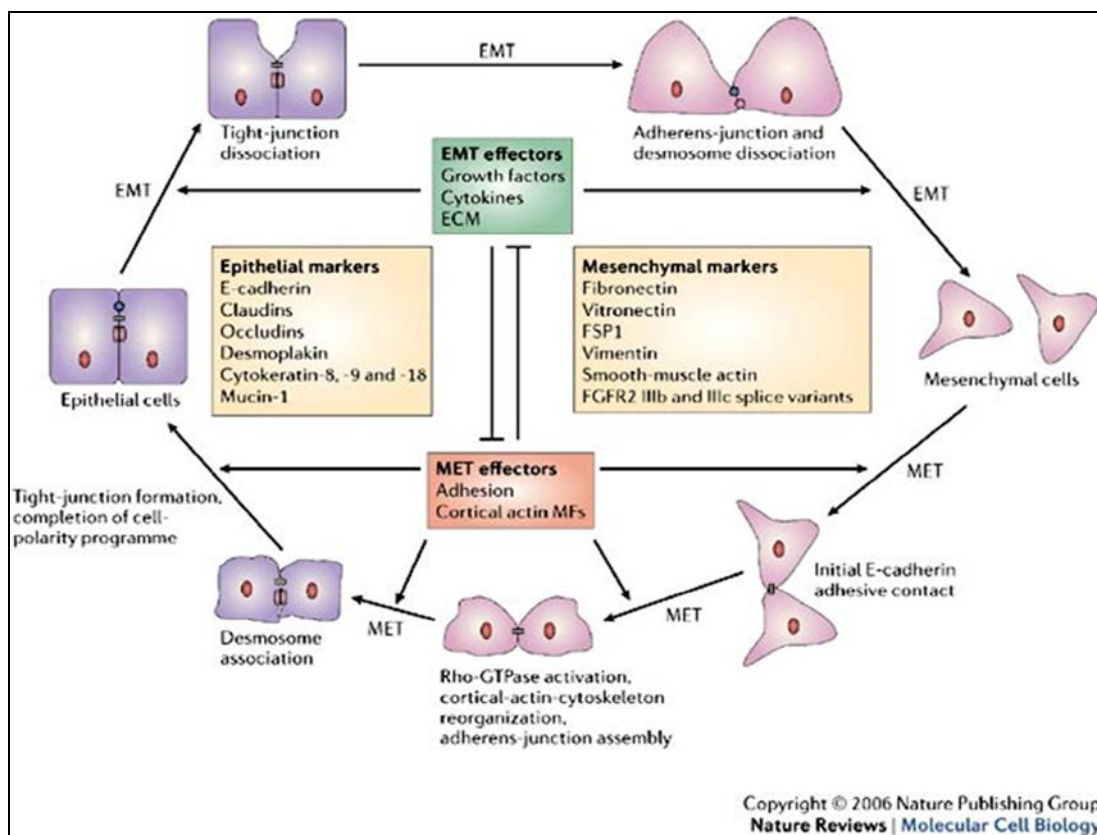

Figure 4. Basic steps in epithelial to mesenchymal transition (EMT), along with the main steps associated with progression of EMT and MET, involved in regulation of adherents and tight junctions. Reprinted from Thiery & Sleeman, Nature Reviews Molecular Cell Biology 2006, with permission from Nature Publishing Group (Thiery & Sleeman, 2006).

1.1.3.4   MST1 in endometrial cancer tumorigenesis

Mammalian Sterile-like 1 (MST1) is a serine/threonine kinase belonging to the Sterile 20-like superfamily, and has been described to be a stress-activated protein involved in a various apoptotic responses (Creasy & Chernoff, 1995)(Minoo *et al*, 2007). It has been identified as a proapoptotic cytoplasmic kinase which comprises a catalytic

domain at the N-terminal, an autoinhibitory region, a dimerization domain and a non-catalytic domain with a nuclear localization motif at the C-terminal (Ng *et al*, 2013)(Creasy *et al*, 1996). When cleaved in the autoinhibitory region, MST1 amplifies the apoptotic signals and produces an extremely active catalytic portion. This process is mediated by cleavage of the caspase-3-recognition motif located between the regulatory and catalytic regions (Minoo *et al*, 2007). The cleaved MST1 passes into the nucleus, where it stimulates chromatin condensation through phosphorylation of histone 2B at Ser14 and thereby apoptosis (Yuan *et al*, 2010)(Praskova *et al*, 2004). MST1 involvement in cell death has also been demonstrated via phosphorylation of FOXO3a-Ser207 and the corresponding site of FOXO1-Ser212 (Lehtinen *et al*, 2006). Other studies have shown that overexpression of MST1 alone can initiate apoptosis via pathways involving activation of SAPK /Jnk, p53, and perhaps other effectors as well(Graves *et al*, 1998)(Lin *et al*, 2002)(Praskova *et al*, 2004).

Phosphorylation of MST1 seems to be essential for regulation of its activity. In the MST1 structure, several phosphorylation sites are located on the threonine residue and have been shown to positively or negatively regulate MST1 activity (Collak *et al*, 2012). Phosphorylation of Thr183 and Thr187 within the N-terminal MST1 activation loop is a crucial molecular event for the activation of MST1 and for cell death in mammalian cells and results in a catalytically more active nuclear form of MST1-N (Minoo *et al*, 2007)(Collak *et al*, 2012).

In addition to its proapoptotic function, MST1 has been shown to be a key factor in cell-cycle progression, tumorigenesis and mammalian development (Cinar *et al*, 2011). Reduced or lost MST1 expression has been seen in HNSCC (Steinmann *et al*, 2009), soft tissue sarcoma (Seidel *et al*, 2007), glioblastoma (Qiao *et al*, 2010), and colorectal cancers (Minoo *et al*, 2007), and is accompanied by a worse prognosis (Seidel *et al*, 2007). Other in vivo studies suggest that conditional ablation of MST1 results in liver enlargement (Song *et al*, 2010). Although a lot has been discovered of the role of MST1 in tumorigenesis MST1 activity in other signaling pathways and in the regulation of invasiveness of endometrial cancer cells remains relatively unexplored.

1.1.3.5 PKN1 in endometrial cancer tumorigenesis

PKN1 (protein kinase N1) also known as PAK1 is a serine/threonine kinase which contain three highly conserved regions: (i) a regulatory domain in the N terminal (ii) a catalytically active part located in the C terminal similar to protein kinase C (PKC) (iii) an area referred to as the D region located between the regulatory and catalytic domains (Metzger *et al*, 2003)(Takahashi *et al*, 1998). The N-terminal domain function is crucial for activation of PKN1 (Takahashi *et al*, 1998) by providing the PKN1 activation loop, which is important for serine/threonine kinase activity and the regions required for interaction with other factors participating in the signaling pathway of PKN1, such as Ro GTPases that bind to the hydrophobic region of PKN1 in the N-terminal (Galgano *et al*, 2009).

Development of the metastatic phenotype requires enhanced cell motility and invasiveness, which necessitates cytoskeletal reorganization. Cdc42, Rac1, and RhoA

small GTPases regulate these processes. Although the exact mechanism of regulation of cytoskeletal changes by GTPases has yet to be clarified, it does involve protein kinase N1 (PKN1) activation (Carter *et al*, 2004). Once activated, PKN1 facilitates downstream signaling actions involved in apoptosis, transformation, cell motility and reorganization of the cytoskeleton. Additional signaling molecules such as JAK2, PDK1 and PKA can also affect PKN1 activity. Diverse signals activate PKN1, resulting in autophosphorylation of numerous sites such as Thr423, which is located in the auto-inhibitory loop of the kinase (Liu *et al*, 2009). PKN1 also activates various signaling pathways, as well as p38 MAPK, JNK, NF-kB and ERKs (Vadlamudi *et al*, 2000).

Kinase-dead mutant of PKN1 inhibits the invasive ability of human breast cancer cell lines (Adam *et al*, 2000), as well as breast epithelial cell migration in response to heregulin (Adam *et al*, 1998). It also inhibits ras-mediated transformation of rat Schwann cells (Tang *et al*, 1998). In rat fibroblasts, PKN1 activation is required for transformation of cells by Rho, Ras, and Rac (Tang *et al*, 1999). PKN1 induces the polarized extension of the actin cytoskeleton in the developing neurite of PC12 cells and also known as a target of downstream network signaling of Rho family GTPases (Daniels *et al*, 1998). Transfection of constitutively active PKN1 in HeLa cells and fibroblasts leads to disintegration of stress fibers and reconstitution of focal structures (Manser *et al*, 1997). Expression of wild-type, constitutively active or kinase-dead PKN1 in NIH 3T3 cells shows substantial differences in actin organization. Expression of constitutively active PKN1 in fibroblasts causes formation of huge polarized lamellipodia at the leading edge that enhance motility and exhibit greater directional movement (Sells *et al*, 1999).

Separate from the explained functions, PKN1 transgenic overexpression in the mammary gland stimulates malignant and premalignant lesion formation in animal models, though with extended latency. Such research is helping to clarify the possible role of PKN1 in tumorigenesis (Ong *et al*, 2011). PKN1 overexpression has been reported to have an association with prostate, colorectal and aggressive ovarian cancers (Carter *et al*, 2004)(Metzger *et al*, 2003)(Galgano *et al*, 2009). Further studies suggested, PKN1 may also influence the invasive characteristics of breast and gastric cancer cells (Adam *et al*, 2000)(Liu *et al*, 2009). Regardless of findings in elucidation of PKN1 function in tumorigenesis, the relevant downstream pathways of PKN1 and its involvement in other signaling pathways and regulation of the invasiveness of endometrial cancer cells remain relatively unexplored.

1.2 TGF-β SIGNALING

1.2.1 TGF-β signaling and endometrial cancer

Transforming growth factor-β (TGF-β), cytokine belongs to TGF-β superfamily has three distinct isoforms including TGF-β1, β2 and β3. Secreted TGF-β is biologically inactive and is referred to as the latent form, L-TGF-β. Biologically active TGF-β forms upon cleavage of the pro-form and dissociation of active TGF-β from latency-conferring proteins, latent TGF-β binding protein (LTBP) and latency-associated peptide (LAP)(Massagué et al, 2000).

Once activated by dimerization, TGF-β mediates signaling pathway using the TGF-β type I (TGF-βRI) and type II (TGF-βRII) receptors. Following binding of TGF-β ligand to TGF-βRII and formation of a heterotetrameric complex with TGF-βRI, TGF-βRI will become phosphorylated by TGF-βRII. However, the TGF-βRI known as activin receptor-like kinase 5 (ALK5), mediates signaling responses in most type of cells; other type of TGF-βRI, such as ALK1, also transduce signals in specific cell types. TGF-βRIs phosphorylate receptor-regulated Smad (R-Smad) proteins for mediating signals: Smad3 and Smad2 proteins by ALK5 and Smad1, Smad5, and Smad8 proteins by ALK1. Upon activation, Smad proteins build a complex with other Smad protein family members referred to as common Smad (co-Smad) and known as Smad4, then move into the nucleus where they mediate activation or repression of transcription of target genes. Other DNA-binding transcription factors assist Smad complexes in regulating high affinity interaction and specificity (Figure 5)(Meulmeester & Ten Dijke, 2011).

TGF-β mediates its signaling through other signaling pathways known as non Smad-dependent TGF-β pathways. There are several alternative TGF-β signaling pathways that do not involve Smad proteins, including phosphoinositol-3 kinase (PI3K), mitogen-activated protein kinases (MAPKs), and Rho-like GTPase. Activation of these pathways is stimulated by non-Smad substrates for either type of TGF-β receptors and further receptor-interacting proteins, such as Par6 and ShcA, that are phosphorylated straight by TGF-β receptor kinases (Mu et al, 2012)(Lee et al, 2007)(Ozdamar et al, 2005).

TGF-β performs a pivotal function in body growth during embryogenesis and in tissue homeostasis. Regulation of such biological processes is mediated by TGF-β signaling, which controls adhesion, invasion, differentiation, proliferation and apoptosis (Heldin et al, 2009). TGF-β functions as a molecule with double role in carcinogenesis. In early-stage cancer, TGF-β has a tumor suppressor role, causing growth inhibition, apoptosis and cell cycle arrest, but in advanced-stage cancer, TGF-β instead promotes tumorigenesis. Cancer cells lose their normal response to TGF-β and develop atypical TGF-β signaling, which promotes cell proliferation, survival, EMT, amplified motility and invasiveness (Jakowlew, 2006).
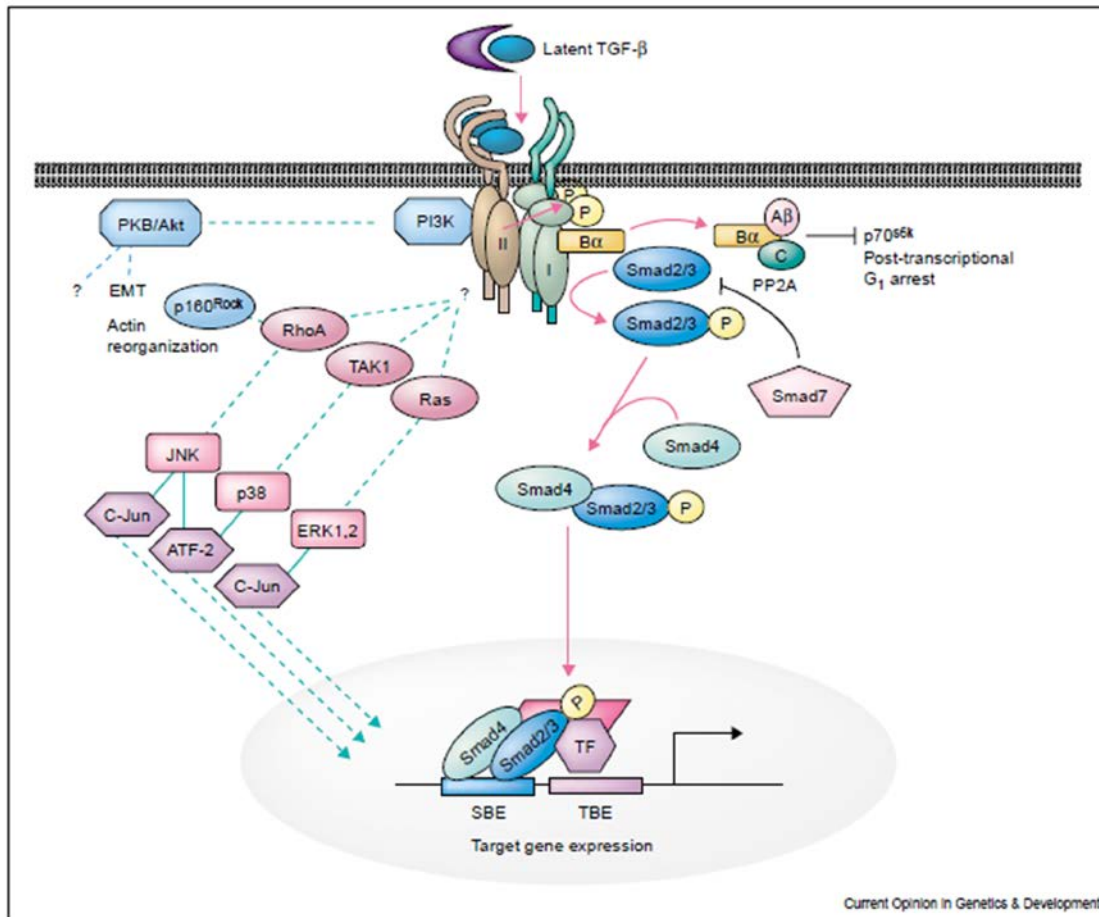
Figure 5. *TGF-β intracellular signaling. After TGF-β ligand binding and formation of a heterotetrameric complex of TGF-βRI and TGF-βRII, R-Smads become phosphorylated by activated TGF-βRI. Phosphorylated R-Smads, principally Smad3 and Smad2, binds to Co-Smad (Smad4) and shift to the nucleus and mediate activation or repression of transcription of the ideal gene in cooperation with further DNA-binding transcription factors. The activated TGF-β receptor complex also mediates signals through activation of other pathways such as PI3K and MAPKs. TGF-β can inhibit the kinase activity of S6 through activation of phosphatase PP2A. However, the molecular details relating to these processes have yet to be explored. Reprinted from Wakefield & Roberts, Current Opinion in Genetics & Development 2002, with permission from Elsevier (Wakefield & Roberts, 2002).*

It has been proposed that TGF-β family members inhibit epithelial cell proliferation and actively participate in neoplastic transformation of human endometrium. Previous studies have suggested that expression of TGF-β isoforms may vary as preneoplastic complex hyperplasia evolves into endometrial carcinoma (Piestrzeniewicz-Ulanska *et al*, 2008). TGF-β has been reported as a paracrine regulator of proliferative activity in human endometrial cells; alterations in the level of expression can promote neoplastic transformation of endometrium (Albright & Kaufman, 1995). Such alterations are not just limited to decreased TGF-β mRNA levels in non-neoplastic tissues compared with endometrial cancer, but also entail variations in expression patterns of specific cells (Perlino *et al*, 1998)(Gold *et al*, 1994)(Parekh *et al*, 2002).

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Alterations in expression level of all three TGF-β isoforms, specifically a substantial increase during the transition from normal proliferative endometrium to simple or complex hyperplasia were prominently observed in the glandular epithelium (Gold *et al*, 1994). However, progression of preneoplastic complex hyperplasia to endometrial carcinoma was not accompanied by any further changes in TGF-β expression. While the level of TGF-β isoform protein expression remained constant in stromal cells, the mRNA expression level increased significantly in stromal cells of complex hyperplasia (Gold *et al*, 1994). These studies propose that one of the early molecular events during neoplastic transformation of endometrium is activation and dysregulation of expression of TGF-β isoforms at either mRNA or protein levels. Dysregulation of the LTBP expression, which is required for TGF-β secretion and proper folding, is also likely to occur, though this has to be yet investigated (Piestrzeniewicz-Ulanska *et al*, 2008).

Although mutations of TGF-β receptor gene are less frequent in endometrial carcinomas but such mutations were frequently observed in pancreatic, head and neck and colorectal cancers (Kim *et al*, 2000). Analysis by Nakashima et al. found that 2.6% of sporadic human endometrial tumors show alterations in the TGF-βRI kinase domain of the gene, while 17% of the analyzed tumors demonstrated sequence changes in the TGF-βRII gene (Nakashima *et al*, 1999).

It has been reported that variations in TGF-βRII affect endometrial cancer progression (Myeroff *et al*, 1995)(Piestrzeniewicz-Ulanska *et al*, 2004)(Ohwada *et al*, 2000)(Sakaguchi *et al*, 2005). Although down-regulation of TGF-β receptors is a well-accepted common characteristic of endometrial cancers, there are still conflicting studies which use different experimental techniques and control groups (Parekh *et al*, 2002)(Piestrzeniewicz-Ulanska *et al*, 2002)(Sakaguchi *et al*, 2005). It has been revealed that TGF-βRII expression is elevated in endometrial cancers with myometrial infiltration, compared with non-infiltrating endometrial cancers (Piestrzeniewicz-Ulanska *et al*, 2004), implying that elevated TGF-βRII expression stimulates local invasion and metastasis (Wakefield & Roberts, 2002)(Derynck *et al*, 2001)(Dumont & Arteaga, 2000).

1.3   TUMOUR BIOMARKERS

Cancer is a heterogeneous disease. Efforts to detect cancer at an early stage have prompted physicians and researchers to develop the concept of biomarker identification (Paul *et al*, 2013). Tumor biomarkers are measurable molecular indicators that are produced in the tumor or other body tissues and fluids in response to the presence of cancer (Tainsky, 2009).

Biomarkers are biological analytes such as nucleic acids (SNPs, chromosome aberrations, DNA copy number changes, differential promoter-region methylation, over- or underexpressed RNA transcripts and regulatory RNAs), proteins (tumor antigens, cell-surface receptors or peptides released into body fluids), lipids, metabolites or biological properties, such as angiogenesis, proliferation, apoptosis, oxygen tension, or clinical characteristics that can be quantitatively measured as indicators of biological processes or therapeutic response in cancer patients (Ludwig & Weinstein, 2005)(Mishra & Verma, 2010).

An ideal tumor biomarker should have high analytical specificity and sensitivity, and be easily, reliably and cost-effectively measurable in a noninvasive or minimally invasive manner (Mäbert *et al*, 2014). Potential biomarkers must overcome several obstacles to be considered for routine clinical use. Biomarkers should be developed in 5 phases: preclinical investigation, clinical assay and validation, retrospective longitudinal and prospective screening and cancer control (Pepe *et al*, 2001).

There are currently several candidate biomarkers to distinguish endometrial tumors, although none are applied in daily clinical routines (Salvesen *et al*, 2012). Recently, studies have reported a few promising prognostic biomarkers, including tumor suppressor P53 (Engelsen *et al*, 2006)(Salvesen *et al*, 1999), DNA ploidy (Pradhan *et al*, 2012)(Susini *et al*, 2007) and progesterone and oestrogen receptors (Creasman, 1993)(Kauppila *et al*, 1986). The majority of such research has involved retrospective analysis of patients who were not subjected to histological subtyping, diagnostic imaging or lymph node biopsy (Salvesen *et al*, 2012). As a result of molecular profiling of primary tumor tissues, several encouraging targets for drug development, such as the FGFR2 and PI3K/PTEN/AKT/mTOR pathway, have been reported (Westin & Broaddus, 2012)(Salvesen *et al*, 2012)(Dedes *et al*, 2011). Thus, such proteins can be used as a companion to help make a diagnosis and select drugs.

1.3.1   Personalized medicine

Heterogeneity in histological appearance, molecular regulatory mechanisms and cellular composition has been found among individual cancer patients (Tian *et al*, 2012)(Saunders *et al*, 2012). Such irregularity results in diverse responses and poses a major drawback to efficacious cancer treatment. A limited response to cancer treatment may entail removal or destruction of some tumor cells, while the tumor may then be repopulated by resistant cells (Saunders *et al*, 2012).

One approach that is gaining momentum for the treatment of various diseases, especially cancer, is personalized medicine (Lin *et al*, 2012). Utilization of different approaches, including genomics, transcriptomics, proteomics and metabolomics, generates a huge quantity of data that may result in the introduction of novel

biomarkers for various purposes. Consequently, further exploration of such studies could be important for the integration of personalized medicine into standard clinical practice and therapy (Lin *et al*, 2012).

Due to inter-patient and intra-tumor variability, the response to any given treatment strategy may vary from one individual to another. Compared with cohort-based studies, molecular profiling of tumors for each patient may become a more effective strategy for cancer management. Personalized treatment aims to maximize treatment benefit for patients while minimizing toxicity and excessive risk, as well as to provide optimal prevention and follow up (Dancik & Theodorescu, 2012). Due to the rapid increase in the number of publications addressing personalized cancer treatment in recent years (Attarha *et al*, 2013)(Zakharchenko *et al*, 2010), treatment of endometrial cancer is also highly likely to benefit from these novel approaches which are quickly gaining momentum (Westin & Broaddus, 2012).

1.4 PROTEOMICS IN CANCER STUDIES

1.4.1 General concept

The word "proteome" was originally coined by Wilkins in 1996 (Wilkins *et al*, 1996). "Proteome" refers to the complete complement of proteins translated by a genome which provides information about 1) translation of expressed gene products 2) the quantities of proteins produced by the gene and 3) the level of posttranslational modification (Humphery-Smith & Blackstock, 1997).

Proteomics is a powerful tool that allows for broad, simultaneous investigation of proteins in complex biological structures and is intended for the evaluation of their expression, function, interactions, structure, modifications and localization. The nature of proteomics enables detection of key pathways for cellular responses to microenvironmental changes by revealing alterations among cellular signaling pathways (Maxwell *et al*, 2011)(Meehan *et al*, 2010)(Creutzberg *et al*, 2000). In addition to the power of proteomics for analysis of the abundance of proteins and their PTMs, this tool has been useful in numerous fields of science, as well as for drug target discovery and identification of novel diagnostic biomarkers (Boja *et al*, 2011).

Subjecting complex biological systems to analysis by proteomics is quickly producing a wealth of 'omics'-scale data requiring additional investigation. The key companion strategy to address these accumulating data is systems biology. Systemic analysis is an effective approach at systems level for investigating the profound nature of biological structures and for visualization of these data to translate them into clear and meaningful biological knowledge (Gehlenborg *et al*, 2010)(Kitano, 2002). Incorporation of proteomic and genomic technologies in combination with cutting-edge bioinformatic tools makes it possible to simultaneously analyze thousands of biological molecules. These techniques allow discovery of novel tumor signatures with enough sensitivity and specificity for early stage detection of cancer, monitoring of disease progression and selection of appropriate treatment, while also paving the way for personalized cancer treatment (Mäbert *et al*, 2014).

1.4.2 Clinical proteomics

Clinical proteomics studies are designed for clinical and analytical validation and application of novel biomarkers for disease treatment and diagnosis, as well as to incorporate the selection, validation, and assessment of the most appropriate and powerful techniques, with the potential for integration with existing analytical platform workflows in clinical laboratories. "Top down" and "bottom up" proteomics are alternative approaches for allocation of targets with potential use in clinical proteomics. In the "top down" proteomics method, unbiased potential targets can be identified by applying high-throughput techniques to cohort or population-based groups and then further analyzed for function, specificity and sensitivity. In the "Bottom up" method, potential targets are selected based on protein–protein or metabolite interaction in known pathways with the aim of identification of extra targets with better stability, sensitivity and specificity (Apweiler *et al*, 2009). Although technological advances in clinical proteomics provide great potential for detection of the causes of cancer, prediction of patient risk for developing specific cancer types, prediction of disease outcome, and guidance for choice of treatment, there are still enormous obstacles to overcome based on previous studies on the clinical application of proteomics (Breuer & Murph, 2011).

The three major factors in proteomics studies are selection of technique for proteins separation, visualization of separated proteins and their precise identification, possibly followed by exploration of post translational modifications (PTMs) and use of bioinformatics for data analysis (Stein & Zvelebil, 2002). Based on different approaches, proteomics include "gel-based" and shotgun proteomics. Mass spectrometry is the preferred approach for protein or peptide identification and characterization (Kolker *et al.,* 2006). A recent advance involves the addition of antibody-based methodology to proteomics. This approach includes application of immunohistochemical analysis (IHC) to tissue microarrays (TMAs), using of antibody arrays for serum-based diagnostic assays and using of reverse phase protein arrays (RPPAs) for pathway analysis (Borrebaeck & Wingren, 2007)(Wingren & Borrebaeck, 2004)(Brennan *et al*, 2010).

1.4.2.1 Gel-based proteomics

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) or two-dimensional electrophoresis (2DE) is the most common methodology in this approach and is one of the cornerstone techniques in proteomics. Proteins are separated based on their isoelectric point (1st dimension, isoelectric focusing or IEF) and their molecular weight (2nd dimension, MW). 2DE is known to be an accepted and powerful technique for proteins separation, followed by protein visualization using Coomassie blue or silver staining. Regardless of the relatively higher sensitivity for detection of proteins in gels, almost all silver techniques have a limited dynamic range (Figure 6)(O'Farrell, 1975).

A fairly recent development within this technique that was introduced by Unlü et al. in 1997 allows differential labeling of various protein samples and separates them on the same gel. In this technology, referred to as differential in-gel electrophoresis (DIGE), protein samples are labeled with fluorescent dyes (cyanine dyes or CyDyes) prior to two-dimensional electrophoresis. The greatest benefit of the DIGE technique

is the application of the same strategy to biological samples, which decreases experimental variations and generates distinct 2DE images from the same gel (Unlü *et al*, 1997).
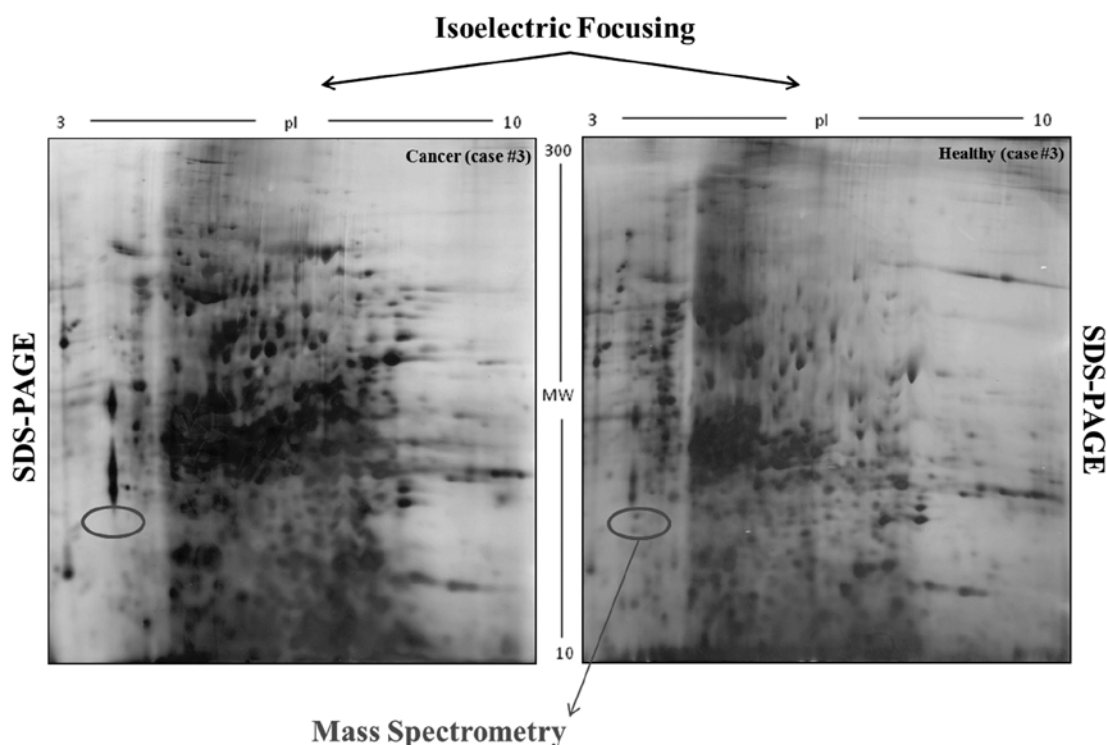

Figure 6. Representative image of 2D gels generated with the proteins extracted from the cancer and healthy adjacent tissue. Proteins are separated based on their isoelectric point (1st dimension) and their molecular weight (2nd dimension).

In order to process the information from the 2DE-gel, translation of gel information into digital data is required. Depending on the staining method, this is carried out using an appropriate imaging system, such as laser-based detectors, CCD cameras or flatbed scanners. Translation of complex data into appropriate biological information is carried out using computerized data analysis provided by software packages developed for this scope.

1.4.2.2 Protein identification using Mass Spectrometry

For most proteomic strategies, mass spectrometry (MS) appears to be an optimal technique for protein identification. Molecular mass of a charged particle is measured by MS by calculating its mass-to-charge (m/z) ratio. In general, mass spectrum is obtained by plotting (m/z) ratio vs. ion abundance (Paul *et al*, 2013).

Basic components of a mass spectrometer are represented in Table 1. Since explanation of the fundamental concepts underlying all available MS technology is beyond the scope of this thesis, we will focus solely on the technique used in our studies: matrix assisted laser desorption ionization-time-of-flight MS (MALDI-TOF-MS).

Sample Introduction	Ionization Source	Mass Analyzer	Ion Detector
• GC Column	• Electron ionization	• Time-Of-Flight	• Electron multiplier
• HPLC Column	• Fast atom bombardment	• Ion trap	• Scintillation counter
• Solid probe	• Laser desorption	• Quadrupole	
	• Electrospray	• Magnetic sector	
		• Ion Cyclotron Resonance and Fourier transform	
		• Orbitrap	

Table 1. Components of mass spectrometer.

MALDI-TOF-MS

Matrix-assisted laser desorption ionization (MALDI) term was invented in 1985 by Hillenkamp and Karas (Karas *et al*, 1985). It has been recognized as a useful source for generation of intact gas-phase ions from a wide array of compounds, including proteins. Ionization by MALDI is achieved by dissolving a protein compound in a solvent, containing in solution crystalline structure of small, organic and UV-absorbing molecules i.e. matrix. Matrix crystals can absorb laser wavelengths similar to those used for ionization of the protein or peptide. Upon UV laser beam irradiation, the matrix crystals become heated through accretion of considerable energy, which excites them and causes analyte ions to pass into a gas phase where they become protonated (acquisition of H^+) and are accelerated by an electric field into the mass analyzer. Although the most frequent ions detected by MALDI are (M^+H^+), in the case of large proteins additional signals for multiply charged ions and oligomeric forms of the analyte have also been detected (Karas & Hillenkamp, 1988)(Figure 7).

The mechanism of ion formation in time-of-flight (TOF) mass analyzers involves pulsed ionization in the existence of an electric field in a short source region. The electric field accelerates the ions into a long field-free drift region. Based on molecular mass, the flying time for the ions to traverse the drift region (TOF) is altered. This process is defined by the relationship: $E={}^1/_2mv^2$, where E is kinetic energy, m is mass of the ion and v is the ion velocity. At a constant energy, ions with higher molecular mass will travel at lower speed, thus have longer time-of-flight than lighter ones. The TOF of the MALDI ions is measured using a clock triggered by a laser pulse. All ions formed from a single laser pulse that are accelerated by the electric field give rise to a transient TOF signal from the detector at the end of the flight tube (Zaluzec *et al*, 1995).
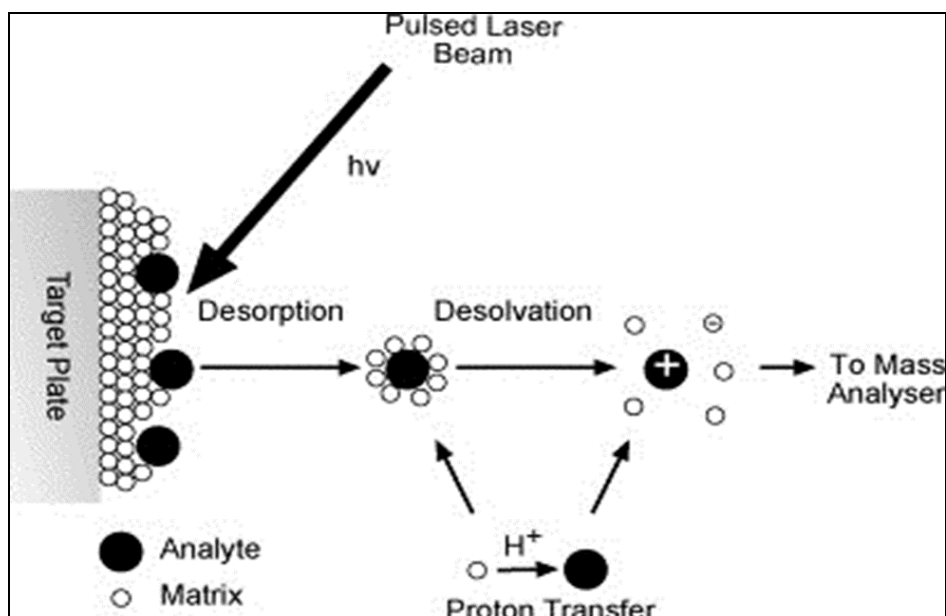
Figure 7. *Matrix-assisted laser desorption/ionization (MALDI) source. Pulsed UV beam targeting the analyte matrix co-crystal drives desorption, followed by desolvation and transfer into the mass analyzer. Reprinted from Kicman AT, Parkin MC, Iles RK, Molecular and Cellular Endocrinology 2007, with permission from Elsevier (Kicman et al, 2007).*

Poor mass resolution was a major weakness of the first TOF analyzers. Several factors that disrupt time of flight of ions with similar mass-to-charge ratio affect final mass resolution. These factors are the kinetic energy distribution, space distribution and time distribution. This issue was significantly overcome with the development of two techniques: 1) delayed pulsed extraction, which introduces a time lag or delay among ion formation in the source field-free region and extraction of ions outside the source by a voltage pulse and 2) The reflectron functions as an ion mirror by turning and directing the ions back through the flight tube (de Hoffmann & Stroobant, 2007). The resulting "spectrum" is the output of the detector, where the y axis corresponds to the ions abundance at a specific point in time (signal intensity) and the x axis represents the "mass-to-charge" ratio (size)(Figure 8).

MALDI-TOF-MS has the advantage of tolerating small quantities of contaminants and has the capacity to analyze small quantities of proteins. MALDI data can be further examined by submitting the data to an automatic database search. The peptide mass fingerprint (PMF) resulting from proteolytic digestion of gel-excised proteins and their mass analysis can be further investigated using protein databases for comparison with theoretical fingerprints of protein sequences (Boja *et al*, 2011).
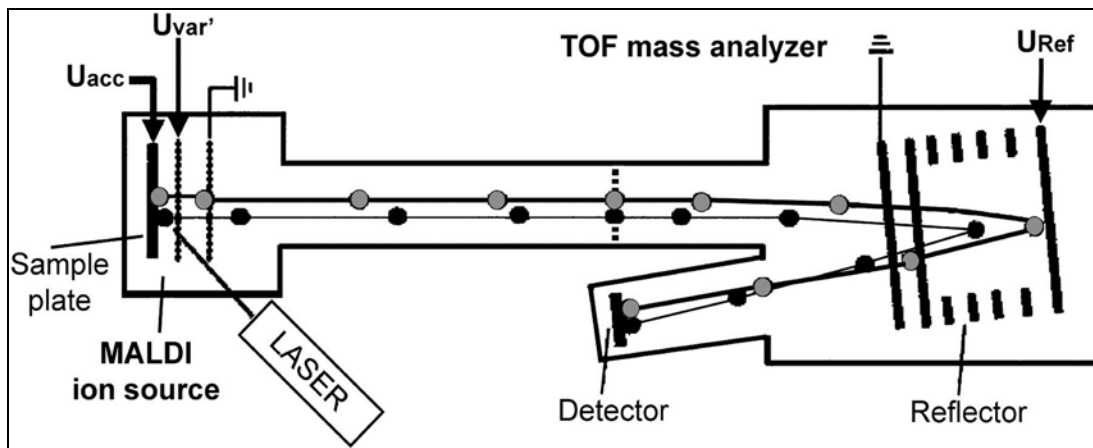
Figure 8. Schematic representation of MALDI-TOF mass spectrometer. Irradiation of a laser beam induces the matrix to become excited which causes analyte ions on the matrix to pass into a gas phase. The existing electric field accelerates the ions into a long field-free drift region. Initial ion energy differences are corrected by reflection of ions in an ion mirror. The detector contains an electron multiplier. With similar kinetic energy in the TOF analyzer, the travel time of ions will be (m/z) ratio of them. URef, ion mirror and reflection voltage, Uvar, applied pulsed to delayed extraction, Uacc, acceleration voltag. Reprinted from Mann et al., Annual Review of Biochemistry 2001, with permission from Annual Reviews (Mann et al, 2001).

1.4.2.3 Gel-free shotgun proteomics

"Bottom-up" proteomics analysis, characterizes proteins by analyzing peptides using proteolysis. The term shotgun proteomics was coined by the Yates Lab and refers to the application of bottom-up proteomics analysis to protein mixtures, thereby indirectly measuring proteins by analyzing the peptides generated from proteolysis of intact proteins. Typically, the peptide mixture in shotgun proteomics analysis is initially fragmented, and then accompanied by liquid chromatography, which is joined to tandem mass spectrometry (LC-MS/MS) for further analysis. In shotgun proteomics, peptides are identified by contrasting tandem mass spectra of fragmented peptides with deposited theoretical tandem mass spectra available in protein databases. Peptide sequences are referenced with proteins to infer the protein molecules. The identified proteins are classified and scored depending on their peptides, since individually, they may refer to one protein or to more than one protein (Zhang *et al*, 2013). The advance represented by this technique made it possible to avoid some of the limitations of gel-based proteomics, including the requirement for a large amount of material, limited dynamic range, low-throughput analysis, difficulties in the identification of acidic, basic, hydrophobic, very small or very large proteins and bias toward abundant proteins (Mäbert *et al*, 2014)(Walther & Mann, 2010).

1.4.3 Systems and network analysis

The complex interactions between elements of biological systems, including DNAs, proteins and metabolites, directly affect activities of such biological entities. Previously, the signaling pathway approach was the only source of knowledge regarding interaction between these components. Exploration of the intricate nature of biological systems involves emergence of several signaling pathways into a higher-

order biological network. The main approach to accomplish this is application of systems and network analysis tools (Lin *et al*, 2012). Acquiring perceptive view of biology at the a system level requires not only evaluation of single cells or part of an organism, but investigation of the structure and dynamics of cellular and organismal function as well (Kitano, 2002).

Recently, promising findings have emerged from several proteomics and genomics studies confirming the effective role of system and network analysis in the exploration of complicated biological systems. These tools make it possible to carry out global mapping of organelles or cells, as well as to find out, conceptualize and analyze the complex function of relevant biological systems. Moreover, analysis of the functional and topological aspects of these networks can help elucidate the cellular regulatory mechanisms responsible for environmental changes (Kwoh & Ng, 2007)(Lin *et al*, 2012).

Interaction networks are built using nodes in various shapes representing genes, proteins, and metabolites, as well as edges representing biological connections (activation, induction, inhibition, PTM, enzymatic-substrate reaction, physical binding) between nodes. The vast majority of biological networks have the characteristic of scale-free network in which a few nodes have the highest number of connections (hubs), while the remainder have just a few connections (Barabási & Oltvai, 2004).

Several tools are available for building and analyzing interaction networks (Gehlenborg *et al*, 2010), such as Cytoscape (Cline *et al*, 2007) and Osprey (Breitkreutz *et al*, 2003), as well as pathway analysis tools such as PathVisio (van Iersel *et al*, 2008) and BioTapestry (Longabaugh *et al*, 2009). Each of these tools has unique functions and can be used in both genomics and proteomics analysis. The preferred tool for our studies was Cytoscape.

Briefly, Cytoscape is a free tool that 1) incorporates proteomics data into a network 2) edits and visualizes the network and 3) analyzes networks using external plug-ins. Graphing tools such as MiMi were used to construct the proteomics data network (Gao *et al*, 2009). Nodes and edges can be selected based on various principles, as well as by attribute or name (Shannon *et al*, 2003). One strong reason for choosing Cytoscape for proteomics analysis is the function that allows use of diverse external plug-ins for systemic analysis. The plug-ins used for systemic analysis in this study are described below. MCODE is an external plug-in used to extract network modules, representing extremely connected regions or clusters in the biological network (Bader & Hogue, 2003). NetworkAnalyzer is a plug-in that calculates various factors related to network topology such as number of connected components and other complex factors, including betweenness centrality and closeness centrality. Knowledge about these parameters enables further investigation of biological properties of the network, including protein-protein interaction and signalling networks (Assenov *et al*, 2008). Centiscape is another topology analysis plug-in that calculates the indexes of centrality for each node and their relationship within the network, which facilitates identification of the crucial regulatory nodes in the network (Scardoni *et al*, 2009).

2 Present Study

2.1 Aims

The general aims of this project are to expand current knowledge about the proteome changes in Endometrial Cancer (EC) with respect to the malignant potential of EC, to identify proteins that are relevant to EC carcinogenesis and to describe potential marker protein patterns that could aid in the diagnosis, evaluation and prognosis of EC.

The specific aims for each paper are described as below:

I. To generate individualized proteome profiles of endometrial tumors to serve as a source of information about the affected molecular processes and to identify key regulators and prognostic signatures of endometrial cancer aggressiveness

II. To explore the involvement of MST1 in regulation of invasiveness of endometrial cancer cells and its contribution to TGF-β and EGF signaling

III. To study the role of PKN1 in invasiveness of endometrial cancer cells and underlying signaling pathways.

2.2 Materials and methods

2.2.1 Materials

Our studies used cultured endometrial cell lines (HEC-1-A, KLE) and human endometrial cancer biopsies. We collected clinical samples at the Department of Women's and Children's Health, Karolinska University Hospital (Stockholm, Sweden), after being granted ethical Permit 2006/649. All materials were obtained at the time of surgery and put on ice prior to processing by a pathologist. The endometrial epithelial tissue samples for the proteomics analysis were stored at -70°C. The sections intended for immunohistopathological diagnostics were obtained at the Department of Oncology-Pathology, Karolinska University Hospital (Sweden).

2.2.2 Tissue sample preparation

Samples for use in the proteomics study were directly extracted in 2D-GE rehydration buffer (2% CHAPS, 0.5% ampholytes, 8 M urea, 0.002% Bromophenol blue, 0.28% DTT, IPG buffer, pH 3-10) and mechanically disintegrated at room temperature using glass beads. We used the supernatants from tissue extracts that were centrifuged for 15 min at 13,000 rpm for 2D-GE.

2.2.3 Two-dimensional gel electrophoresis

We carried out 2D-GE in two steps. We used an IPGPhor unit (GE Healthcare, Uppsala, Sweden) to conduct IEF as follows: 20 µA per strip, rehydration at 50 V; 3 hrs, 1000 V; 1 hrs, 5000 V; 10 hrs, or until 32,000 Vhrs was reached. Following IEF, we equilibrated IPG strips in 2 stages using equilibration buffer (1.5 M Tris-Hcl, pH 8.8, 30% glycerol, 2% SDS, 6 M urea, 0.002% Bromophenol blue) with 1% DTT for 10 min in first stage, and with 2.5% iodoacetamide for 10 min in the second. We conducted second dimension SDS-PAGE using an Ettan Dalt Six electrophoresis system (GE Healthcare, Uppsala, Sweden) as follows: 0.5 W per gel for 15 min, 1 W per gel for 30 min, and 10 W per gel until the run was completed. For each sample, we produced two 12% gels. Proteins were detected using 0.25% silver nitrate.

2.2.4 Gel image analysis

Stained gels were scanned and the spots were analyzed by tool embedded in the dedicated software Image Master Platinum v6.0 (GE Healthcare, Uppsala, Sweden). We ensured the statistical significance ($p<0.05$) of the spot selection using the Student's t-test embedded in the software. Proteins that demonstrated either a > twofold change in expression pattern, or that exhibited a unique pattern of expression between the tumor and adjacent histologically normal tissue for each pair of cancer/non-cancer tissue samples were considered for mass spectrometry identification.

2.2.5 Mass spectrometry

After excision of protein spots from gels they were destained and digested in-gel with trypsin (modified sequence-grade, porcine, Promega, Madison, WI, USA) as described earlier (Attarha *et al*, 2013). We desalted and concentrated tryptic peptides on a µC18 ZipTip (Millipore Billerica, MA, USA). Fifty percent acetonitrile, with a matrix α-Cyano-4-hydroxycinnamic acid was used to elute peptides onto the MS target and subsequently analyzed using a MALDI-TOF Reflectron (Waters, Milford,

USA). We processed the mass spectra using Micromass software (MassLynx™ Software v4.0). Autolytic peptides produced from exposure to trypsin (842.510, 1045.564 and 2211.105 Da) were used for internal calibration of peptide spectra. For protein identification, the ProFound search engine (http://65.219.84.5/service/prowl/profounf.html) was used to search the NCBI nr sequence database. Partial oxidation of methionine, alkylation with iodoacetamide and one missed cleavage were acceptable. We limited the search parameters for Mr and pI by comparing the migration position of protein in generated gels and a mass tolerance of less than 0.1. The species search was set to "Homo sapiens." Z-value, sequence coverage, mass precision and probability value of the matched peptides were used to evaluate the significance of the identified proteins.

2.2.6 Systematic analysis

We converted the protein names into GO terminology (http://biodbnet.abcc.ncifcrf.gov/). The data were subjected to systematic analysis using Cytoscape and GoMiner (http://discover.nci.nih.gov/gominer/) software. Identified proteins were classified into biologically coherent categories and assessed by GoMiner. Cytoscape was used to explore relationships between the identified proteins. Analysis was conducted by generating a network using the proteins we identified, the MiMIplugin, along with all available databases. We viewed the network in Cytoscape; the Network analysis plugin was used to calculate betweenness, after which the AllegroMCODE plugin was used to extract network modules. When determining network connectivity we used Fisher's exact test for calculating p-value.

2.2.7 Immunohistochemistry

Expression of targeted proteins was evaluated using IHC analysis of EMC1021 (Pantomics Inc., Richmond, CA, USA) and UT501 USBiomax (US Biomax Inc., Rockville, MD, USA) EC arrays as described earlier (Attarha *et al*, 2013). Of the 50 cases on the UT501 array, 5 cases were normal, 2 were serous adenocarcinoma, 41 cases were endometrioid adenocarcinoma and 2 were representing clear cell adenocarcinoma. The other array included 97 cases of EC along with 5 cases of normal tissue. Arrays were stained with target primary antibodies at dilution recommended by the manufacturer. DakoCytomation Target Retrieval Solution, High pH (DAKO, Carpinteria, CA, USA) was used for antigen retrieval. We stained the sections with VECTASTAIN Elite ABC kits (Vector Laboratories Inc., Burlingame, CA, USA), then counterstained using hematoxylin, after which we mounted using Fluoromount G (Southern Biotechnology, Birmingham, AL). We photographed and analyzed the slides with a Leica DFC camera and embedded software Leica QWin Standard (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

2.2.8 Immunoblotting

We resolved cell lysates on 10% SDS polyacrylamide mini-gels and subsequently transferred them to nitrocellulose membranes (Whatman, protran, Dassel, Germany), then blocked the membranes with 5% Bovine serum albumin (BSA) and incubated with primary antibodies using, the dilution specified by the supplier, and culminated the process by incubating with HRP-conjugated secondary antibody (GE Healthcare,

Uppsala, Sweden). Luminol Reagents (Santa Cruz Biotechnology Inc.) were used for protein visualization.

2.2.9   Construct and transfection

Drs. Hideyuki Mukai and Zengqiang Yuan kindly provided us with expression constructs for targeted proteins. HEC-1-A cells in 12-well plates were transfected with GeneJuice® transfection reagent, in accordance with the manufacturer's recommendations (Novagen, Darmstadt, Germany), in order to generate stable transfected cell lines. Following transfection for 48 hours, we transferred the cells onto a 10-cm petri dish and subjected them to selection for 3 weeks using 200µg/ml of G418. The single colonies we picked up were culture-expanded for 4 weeks. Immunoblotting with specific antibodies was used to validate protein expression within stable transfected cells. Because the cells did not respond to identical transfection conditions or take up DNA following repeated passage, we chose to generate transiently transfected cell lines instead.

2.2.10 Cell Culture

Our studies used the HEC-1-A and KLE cell lines, which were ordered from ATCC (Manassas, VA). Selection of a media for cell culture was based on the manufacturer recommendation, supplemented with 10% FBS.

2.2.11   Cell proliferation assay

We measured proliferation activity of cells with the CellTiter 96® AQueous One Solution Cell proliferation assay (Promega, Promega Biotech AB, Stockholm, Sweden). The assay performed in accordance with the recommendations of the manufacturer. McCoy's 5A Medium Modified supplemented with 1% penicillin/streptomycin and 10% FBS was used to grow the cells.

2.2.12   Cell apoptosis assay

The Cell Death Detection ELISAPlus (Roche, Germany) was used to assess cell apoptosis. We placed cell lysates on a streptavidin-coated microplate and supplemented it with a combined solution of anti-DNA-peroxidase (anti-DNA-POD) and anti-histone-biotin, followed by incubation at 25˚C for 2 hrs. Following the washing step and removal of unbound antibodies photometric determination of POD was carried out at 405 nm using ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) as substrate.

2.2.13 Wound healing assay

We grew cells in 10% FBS culture medium for 48 hours until they reached confluence. The confluent culture monolayers were lightly scratched using a 20 µl-pipette tip, and images under light microscopy were obtained from the scratched areas. After incubation overnight, a new set of images of the scratched areas were obtained. TScratch software was used for quantification and involved measuring the open wound area, defined as the fraction of open area at the later time point compared with its earlier initial appearance, expressed as a percentage (Gebäck et al, 2009).

2.2.14 Migration assay

We seeded the cells suspended in culture medium onto the well membranes of the 96-well ChemoTx® chemotaxis system plate (cat. no. #116-8; Neuro Probe Inc.). After incubating for 24 h, we washed the membrane twice with PBS and used 70% ethanol as a fixative. A cotton swab was used to remove non-migrated cells from the upper side of the membrane, after which we stained the membrane with 0.5% crystal violet and used ImageJ software for visualization and quantification (Schneider *et al*, 2012).

2.2.15 Invasion assay

We seeded the cells suspended in culture medium onto the 3% gelatin-covered membranes of the 96-well plate of the ChemoTx chemotaxis system (cat. no. #116-8). Then, after 24 h, we washed the membranes two times in PBS and used 70% ethanol for fixation. A cotton swab was used to remove the non-invaded cells from the upper side of the membrane. Finally, we stained the membrane with 0.5% crystal violet, used ImageJ software for visualization and quantification (Schneider *et al*, 2012).

2.2.16 Statistical analysis

We used the Mann-Whitney test to evaluate statistical significance of differences observed in unpaired groups and the Kruskal-Wallis test followed by Dunn's Multiple Comparison Test for multiple groups. For statistical analysis we used Graph Prism 6 software (GraphPad Software, San Diego, CA) and p-value<0.05 was considered significant.

2.3　Results and Discussion

2.3.1　Paper 1

Individualized proteome profiling of human endometrial tumors improves detection of new prognostic markers

Proteomic profiling of tumors may improve our ability to diagnose and treat cancer. Researchers often search for possible biomarkers by examining data shared by a multitude of patients, without concerning themselves about variations that may distinguish one patient from another. However, since major researches of human carcinogenesis have recently presented pronounced differences in tumor-related profiles among individuals (Saunders *et al*, 2012)(Tian *et al*, 2012), exploring personal tumor variables is crucial for a deeper comprehension of tumorigenesis, diagnosis and therapeutic choices.

Concerns about making a diagnosis of endometrial cancer based on histopathology necessitate identification of additional biomarkers to describe the functional molecular profile of endometrial tumors. Despite numerous attempts, we have as yet been unable to identify any molecular biomarkers that reflect aggressive behavior of individual endometrial tumors (Salvesen *et al*, 2012). Recently, studies have reported a few promising prognostic biomarkers, including tumor suppressor P53 (Engelsen *et al*, 2006)(Salvesen *et al*, 1999), DNA ploidy (Pradhan *et al*, 2012)(Susini *et al*, 2007) and progesterone and oestrogen receptors (Creasman, 1993)(Kauppila *et al*, 1986). The majority of such research has involved retrospective analysis of patients who were not subjected to histological subtyping, diagnostic imaging or lymph node biopsy (Salvesen *et al*, 2012). As a result of molecular profiling of primary tumor tissues, several encouraging targets for drug development, such as the FGFR2 and PI3K/PTEN/AKT/mTOR pathway, have been reported (Westin & Broaddus, 2012)(Salvesen *et al*, 2012)(Dedes *et al*, 2011). Thus, such proteins can be used as a companion to help make a diagnosis and select drugs.

Regulatory proteins are less likely to be identified than high abundance proteins, since individual tumors display substantial variation. Traditionally, identification of tumor-specific changes involves listing the proteins that are universally expressed by all tumors, and then formulating a second list of proteins universal to cancer-free controls. A comparison of these lists reveals differences in protein profiles that may be interpreted to reflect significant cancer-specific changes. However, this strategy presumes that tumorigenesis proceeds along a common pathway, demonstrating identical changes in every case of cancer, but since tumors are heterogeneous, this strategy may overlook individually unique tumor profiles. Our study addresses the previously overlooked individual characteristics of endometrial cancers, which could serve as a wellspring of information regarding specific molecular processes; regulators of such processes could potentially be useful for predicting aggression in individual endometrial tumors.

We used a two-component approach to assess how individual characteristics of endometrial tumors contribute to tumorigenesis. First we conducted proteome profiling for three separate cases. Next we carried out a meta-analysis of individual endometrial tumor characteristics, which served as a basis for finding potential biomarkers. The procedure also involved IHC validation of a separate set of EC cases. Because we concerned ourselves with the role of individual tumor characteristics, we had to confine ourselves to a given number of cases for the profiling portion of this study. To validate the markers chosen from our proteomics results, we conducted tissue microarray testing on a total of 168 EC and normal cases. Since we uncovered intact proteins in tumor sampling, we studied these instead of examining peptides that were produced artificially, as those used in peptide-based strategies with tandem mass spectrometry. Using 2D gel electrophoresis, we were able to identify over 2,000 intact proteins with their isoforms in each run.

The three tumors demonstrated substantial variation in protein expression. We identified 298 tumor-related proteins in case one, and 121 and 165 tumor-related proteins in cases two and three, respectively. Considerable variation in tumor proteins was found when analyzing these three cases. For each tumor, we conducted molecular profiling on the proteins discovered during proteome profiling in order to uncover the implications of this variability.

The identified proteins were used to construct networks for each case of endometrial carcinoma in order to map the molecular profiles of each tumor. Next we used our networks to pinpoint what signaling mechanisms were influenced in the tumors. Researchers have definitively shown that the various components of a specific signaling network are able to regulate important cell functions in an identical way (Souchelnytskyi, 2005). We postulate that various proteins may be impacted in the individual tumors of different patients, but the impact of these proteins on basic cell functions may still be similar. Consequently, we tested the concept that "many means lead to the same end" by studying the proteins that were uncovered on a case-by-case basis, and finished by subjecting individual datasets to meta-analysis in order to uncover shared mechanisms.

Our initial task was to find out which of the identified proteins of a particular tumor impacted what functional domain. Next, we compared these functional domains related to each case among the three cases of endometrioid carcinoma. As anticipated, we found that functional domains overlapped significantly among these three cases of endometrioid cancer. Highly overlapping domains involve regulation of proliferation and cell growth, immune response, cell migration, hormonal response and control of angiogenesis. Overlap indicates similarities between the impacted tumor mechanisms. But, when analyzing individual network components, no overlap was found, suggesting that individual tumors employ different mechanisms to realize similar objectives, such as uncontrolled growth. This finding is significant for selection of cancer treatment. Different drugs can be used to target the unique properties of each tumor. In other words, a different drug may uniquely useful for each of our three cases—methotrexate in case one, salinomycin in case two, and trichostatin A in case three—which underscores how attention to individual tumor characteristics may be highly relevant when formulating individualized cancer treatment.

To confirm the usefulness of the proteins we identified and their signaling networks to serve as predictive biomarkers of individual tumor aggression, we expanded our focus to include both aggressive and non-aggressive signatures through extraction of common dependencies to the protein networks of these three cases, and for species stored in the Array Expression database of EBI. The correlation between gene expression data and proteome profiling that we noted is highly significant because it validates our own proteome profiling of only three cases, which discovered changes also found in our large-scale study. This encouraging finding underscores the need for further research on the proteins we identified here as biomarkers of endometrial cancer invasiveness and aggressiveness.

We created interaction networks among proteins representing invasive and non-invasive characteristic and the proteins and genes on which they may have an impact and the genes validated by analyzing published datasets to discover systemic properties of the signaling mechanisms of these proteins. We concentrated on invasiveness, because it is a prerequisite for tumor aggression.

When addressing aggressive and non-aggressive tumor behavior, we anticipate that the main intersections among nodes of non-invasive and invasive networks could be essential regulators of aggressiveness. By analyzing node connectivity we found that ACTA1, ZBTB16, TAF1, HNF4A may serve as important regulators for the non-aggressive network, while TAF1, HNF4A, JUN, ATF7IP, ATF2 were uncovered as possible aggressive network regulators.

We chose PKN1 and MST1 to validate by tissue microarray. Our choice of these proteins was motivated by published data possibly implicating them in EC (Ng *et al*, 2013)(Galgano *et al*, 2009). A validation study was conducted on the same cases that we subjected to proteome profiling and on other endometrial cancer cases and performed a tissue microarray on the endometrial cancer specimens. We subjected a total of 168 cases to analysis using two different tissue microarrays. We screened individual tumor profiles to pinpoint these two proteins and our validation study and confirm their potential as predictors of aggressive tumor behavior in endometrial cancer.

The most important barrier to developing efficacious cancer therapy is the variability of molecular profiles displayed by different tumors. We pursued a strategy of subjecting three endometrioid carcinoma cases to complete proteomic profiling and systematic study in order to reveal the unique properties of each tumor. We then carried out meta-analysis of the individual profiles using the proteome profiling results for each tumor. Breast tumors have been profiled using similar strategies, which has significantly improved understanding of the mechanisms that are involved in tumorigenesis (Zakharchenko *et al*, 2010). Our validation study, based on immunohistochemistry, investigated a large cohort of cases and was able to confirm the utility of this strategy for the study of EC.

Frequently observed features common to a number of cancers, including endometrial, are molecular regulatory mechanisms and cellular composition, as well as inter-

patient and intra-tumor variability in histological appearance (Saunders *et al*, 2012)(Tian *et al*, 2012). It is precisely such variability that creates a significant barrier to efficacious treatment for cancer. The high rate of incomplete treatment response can likely be attributed to partial clearing of tumor cells, which permits more resistant cells to rebuild the tumor (Saunders *et al*, 2012). Gene mutation and RNA expression studies are the principle tools of tumor molecular profiling (Kohlmann *et al*, 2012). However, endometrial cancer proteome profiling is on the rise and our data support the successful use of proteome studies to improve diagnostics. The combination of 2D-GE and MS has proven to be the best intact-protein proteomics approach to investigating full-length proteins, thereby yielding a genuine protein-based profile (Wilkins *et al*, 2006).

A combined approach using the various OMICs studies has been integral to achieving a significantly more complete overview of the mechanisms than any single technology could achieve (Koboldt D, 2012)(Tian *et al*, 2012). The optimal basis for formulating cancer diagnostics and therapy would be a combined approach using proteomics, metabolomics, transcriptomics, genome sequencing and clinical observations. The suitability of combining proteomics and transcriptomics has been demonstrated by our results, a combination that verifies the pertinence of our proteomics findings. Nevertheless, the need for more detailed protein ontology along with incomplete knowledge of genes from transcriptomic studies have resulted in missing data, which now poses a challenge (Lan *et al*, 2003). Systems biology tools may solve this problem in part, since they allow us to explore interdependencies among identified genes and proteins (Hucka *et al*, 2003). The most common strategy to explore such dependencies is to create a network, which is also helpful for predicting key regulators. Network topology provides us with a computer-assisted approach to help identify key functions and associated regulators, thereby improving quality and adding to the findings significance (Hucka *et al*, 2003). We were able to identify PKN1 and MST1 as possible endometrial tumorigenesis regulators by studying protein networks using Cytoscape and by combining mRNA expression profiles with proteomics data.

The possible tumorigenic role of the PKN1 and MST1 kinases has already been demonstrated. MST1 has been demonstrated to promote growth of hepatocellular carcinoma in the presence of downregulation of NORE1B (Ng *et al*, 2013). Research has shown that loss of cytoplasmic MST1 expression is a tumor progression marker in colorectal cancer (Minoo *et al*, 2007). Research has correlated PKN1 upregulation with prostate, colorectal and aggressive ovarian cancers (Carter *et al*, 2004)(Metzger *et al*, 2003)(Galgano *et al*, 2009). Other research implicates PKN1 effect on the invasive characteristics of breast and gastric cancer cells (Adam *et al*, 2000)(Liu *et al*, 2009). Although recent research has helped to elucidate the mechanisms by which PKN1 and MST1 are involved in tumorigenesis, we have yet to investigate the role of these proteins in progression and development of EC. The potential of PKN1 and MST1 to serve as potential predictive biomarkers of EC, with sufficient sensitivity and specificity to be useful in a clinical setting, is suggested by our data.

The mounting perception that there may be up to 100 molecular profiles in cancer has now sparked further studies aimed at elucidating the individual properties of tumors

(Koboldt D, 2012)(Tian *et al*, 2012)(Souchelnytskyi, 2005). Our study exemplifies such tactic. Our data indicate that complete profiling of individual tumors may pave through targeting a specific tumor in an individual patient through customized cancer drug treatment. Our approach may help us to use truly personalize treatment for cancer treatment.

2.3.2 Paper 2

Mammalian sterile-like 1 kinase inhibits TGFβ and EGF-dependent regulation of invasiveness, migration and proliferation of HEC-1-A endometrial cancer cells

Tumorigenesis is the result of malfunction of many genes, proteins and metabolites. Among these molecules, TGF-β and EGF have prominent places as strong regulators of tumorigenesis. TGFβ pathway associate with several signaling pathways including epidermal growth factor (EGF) signaling pathway to modulate its effects (Dunfield & Nachtigal, 2003). TGF-β and EGF intracellular signaling involves sharing intracellular signaling mechanisms. The extensive cross-talk between TGF-β and EGF involves proteins and genes such as Smads, Erk1/2, p38 and PI3K; new cross-talk components are now being explored.

Mammalian Sterile-like 1 (MST1) is a serine/threonine kinase belonging to the Sterile 20-like superfamily, and has been described to be a stress-activated protein involved in a various apoptotic responses (Creasy & Chernoff, 1995)(Minoo *et al*, 2007). MST1 functions as a key regulator of mammalian development, cell-cycle progression and tumorigenesis (Cinar *et al*, 2011). A loss or reduction of MST1 expression has been suggested in head and neck squamous cell carcinoma (Steinmann *et al*, 2009), soft tissue sarcoma (Seidel *et al*, 2007), glioblastoma (Qiao *et al*, 2010), and colorectal cancers (Minoo *et al*, 2007), along with worse prognosis in cancer (Seidel *et al*, 2007). Other in vivo studies have also indicated that conditional ablation of MST1 resulted in liver enlargement (Song *et al*, 2010). Despite recent advances in our understanding of the role of MST1 in tumorigenesis, involvement of MST1 in other signaling pathways and in regulation of invasiveness of endometrial cancer cells remains relatively unexplored. We report here that MST1 modulates cross-talk between TGFβ and EGF in regulation of cell proliferation, migration and invasiveness.

We have previously identified MST1 as a protein that is deregulated in endometrial cancer (Attarha *et al*, 2013). Network analysis suggests that MST1 may be involved in cross-talk between TGFβ and EGF. The involvement of MST1 may have an impact on cell proliferation via regulation of Ras and on cell death via regulation of caspase-3 and p53. It should be noted that the molecular mechanisms of MST1 action are undergoing extensive exploration, and we may therefore expect missing interactions between MST1, TGFβ and EGF. Consequently, to explore the role of MST1 in cellular response to TGFβ1 and EGF, we used overexpression of wild-type and the Ser82Ala mutant of MST1 in human endometrial carcinoma cells (HEC-1-A). The mutant MST1 was reported to have strongly decreased phosphorylation and disrupted dimerization (Bi *et al*, 2010). Both MST1 constructs were expressed at similar levels, and expression was not affected by treating the cells either with TGFβ1 and EGF alone or in combination. This indicates that TGFβ1 and EGF did not affect the stability of the MST1 expressed in HEC-1-A cells. MST1-transfected cells showed no signs of enhanced cell death, indicating that MST1 itself under the conditions used

did not induce cell death. We were therefore able to use HEC-1-A cells transfected with wild-type and mutant MST1 for further study.

First, we explored whether MST1 might affect cellular physiology and response to TGFβ1 and EGF in regulation of cell proliferation, apoptosis, migration and invasiveness. To study cell proliferation we performed MTT assay. We observed that TGFβ1 and/or EGF reduced the proliferation rate of cells. The inhibitory effect of EGF was unexpected, but also reproducible. The MST1 effect was rather marginal when the cells were treated with TGFβ1 or EGF alone. The wild-type MST1 inhibited MTT activity when expressed in non-treated cells, but this effect was not observed when cells were treated with TGFβ1 or EGF. The most pronounced inhibitory effect of combined treatment with TGFβ1 and EGF was prevented in cells expressing WT or mutant MST1. One interesting observation is that the MST1 mutant had an impact similar to that of the wild-type construct, indicating that impairment of MST1 activity is not essential for MST1 activity to have a negative impact on TGFβ1 and EGF cross-talk.

One cellular mechanism affected in cancer is cell death. We therefore studied whether MST1 kinase affects cell apoptosis. Expression of MST1 promoted apoptosis activity of cells when treated with EGF and TGFβ1, while a stimulatory tendency was noted in non-treated cells. However, these effects were not strong, and did not affect cellular growth. We therefore concluded that the effect of MST1 on cell death was not pronounced.

To explore the effects of MST1 on cell migration, we performed wound healing and membrane migration assays. These assays explore cells in different conditions: in a confluent monolayer and in a sparse culture. However, in both assays the cells are prompted to migrate and this migration is then measured. The wound-healing assay showed that expression of MST1 constructs inhibited cell migration in non-treated and TGFβ1 or EGF-treated cells. When cells were treated with both TGFβ1 and EGF, significant inhibition was observed. This inhibition was slightly counteracted by expression of wild-type MST1. The most pronounced effect of TGFβ1 and EGF treatment on cell migration was observed in a membrane migration assay. We observed that combined treatment with TGFβ1 and EGF strongly induced cell migration, while treatment with either alone had only a marginal effect. Expression of both MST1 constructs resulted in much lower cell migration. Interestingly, the ability of MST1 to dimerize was not important for this inhibitory effect. Another conclusion is that cells in a dense monolayer may respond differently to stimulation of migration than cells in a sparse culture.

Cell invasiveness is an important characteristic for tumorigenesis. We explored invasiveness of cells through a layer of denatured collagen (gelatin). We observed that transfection of MST1 constructs enhanced cell invasiveness, although the level of induction varied with different treatment conditions. The strongest stimulatory effect was observed with expression of mutant MST1 in non-treated cells. This stimulatory effect disappeared when cells were treated with TGFβ1 and/or EGF. We observed that combined treatment of cells with TGFβ and EGF decreased invasiveness when MST1 constructs were expressed. This observation indicates that abrogation of

dimerization and subsequent inhibition of the kinase activity of MST1 was not essential when cells were treated, but were important for response of non-treated cells. Thus, MST1 constructs counteracted the stimulatory effect of combined treatment with TGFβ1 and EGF on cell invasiveness.

TGFβ and EGF employ many different signal transducers, with convergence on many common targets. To explore at what level of the signal transduction MST1 may interfere with TGFβ and EGF signaling, we measured expression and/or activation of Smad2, pRb, FAK and Erk1/2, as well as expression of vimentin and E-cadherin.

Phosphorylation of Smad2 at its C-terminal serine residues reflects activation of signaling downstream of TGFβ receptors (Souchelnytskyi *et al*, 1997). We observed that only expression of wild-type MST1 had a significant inhibitory effect on expression of Smad2 and Smad3, and phosphorylation of Smad2. Under all other conditions, variations were less pronounced. This indicates that MST1 does not affect proximal TGFβ signaling events via Smad2 protein.

We observed no significant effect of MST1 on pRb expression nor its phosphorylation on Serine 780 residue. This observation is not in line with the results of the MTT proliferation assay, and suggests that pRb expression and Ser780 phosphorylation do not correlate with MST1 impact. Phosphorylation of Erk1/2 kinase often correlates with cellular proliferation rate. As in the case of pRb, we see no correlation between Erk1/2 phosphorylation and the results of the proliferation assay. However, we observed that expression of MST1 constructs did modulate Erk1/2 phosphorylation when treated with TGFβ1 and/or EGF, suggesting that Erk1/2 phosphorylation is modulated by MST1, but its impact on cell physiology remains to be elucidated. At least the impact is not reflected in the cellular proliferation rate.

Phosphorylation and expression of focal adhesion kinase (FAK) allows monitoring of cytoskeleton rearrangements involved in cell migration. We observed that FAK phosphorylation correlated with enhanced invasiveness of HEC-1-A cells, whether transfected or not, that were treated with different combinations of TGFβ1 and EGF. For migration assay results, correlation was observed for all conditions, except among non-transfected cells, which showed high phosphorylation of FAK, while no migration through the membrane was observed.

E-cadherin and vimentin are markers of the epithelial-mesenchymal phenotype of cells. They are also used as markers to evaluate invasiveness-related epithelial-mesenchymal transition (EMT). HEC-1-A cells show detectable expression levels of vimentin and E-cadherin. Expression of either of the MST1 constructs did not modulate vimentin expression, while expression of the wild-type MST1 reduced E-cadherin levels following single TGFβ1 and double TGFβ1 and EGF treatments. TGFβ-dependent inhibition of E-cadherin expression is known to be a part of TGFβ-induced EMT. The results of immunoblotting for E-cadherin indicate that MST1 has modulatory impact on TGFβ and/or EGF regulated expression of E-cadherin, but this impact must be combined with other regulatory processes, which then would result in impact on cell proliferation, migration and invasiveness. Among the markers of

intracellular signaling pathways that we evaluated, phosphorylation of FAK showed good correlation with a pattern of cellular invasiveness.

Cellular functions are controlled by combinations of different regulators. Here we described the impact of MST1 on functional interaction between TGFβ and EGF in the regulation of cell invasiveness, migration and proliferation. Recent studies showed that MST1 regulates cell death, differentiation and proliferation (Qin *et al*, 2013). Aberrations in MST1 expression have been observed in tumorigenesis, with indications that MST1 may have a tumor suppressive role (Ng *et al*, 2013)(Minoo *et al*, 2007). Many laboratories are currently investigating MST1 intracellular signaling mechanisms; one key conclusion is that MST1 may play the role of coordinator between different pathways.

EGF and TGFβ are two well-studied regulators of tumorigenesis. EGF is predominantly a tumor-promoting factor, presumably due to its strong pro-mitogenic activity (Scaltriti & Baselga, 2006). In contrast, TGFβ is a strong inhibitor of epithelial cell proliferation. However, TGFβ has a dual role in tumorigenesis (Katsuno *et al*, 2013a)(Massagué, 2012). In the early stages, it prevents tumor growth, while in later stages of cancer it stimulates metastasis. Extensive cross-talk between EGF and TGFβ has been described. Intracellular regulators that were first considered to be specific to TGFβ or EGF pathways, were later shown to be shared between these pathways (Katsuno *et al*, 2013a)(Massagué, 2012).

The identification of MST1 as a protein with potential involvement in the cross-talk between TGFβ and EGF prompted us to explore whether MST1 can indeed affect EGF and TGFβ-dependent regulation of cell invasiveness, migration and proliferation. MST1 has been reported to induce cell death (Graves *et al*, 1998)(Qiao *et al*, 2010)(Lin *et al*, 2002). We observed only marginal cell death induction with transfection of wild-type MST1 in HEC-1-A cells. Our results showed that MST1 may act as a negative regulator of the combined action of TGFβ and EGF on cell invasiveness and migration, while it has no pronounced effect when cells are challenged with each of the growth factors independently. This observation underscores the importance of exploring combination treatments. The challenge of such exploration lies in the high number of intracellular regulators that would need to be tested. We monitored some of the proteins that may be involved in activation of TGFβ and EGF signaling (Smad2 and Erk1/2) and other proteins that may reflect migratory and invasive mechanisms (FAK, vimentin and E-cadherin). The exploration of cellular responses to the combination of TGFβ, EGF and MST1, along with the evaluation of protein markers of signaling pathways reported here, provide incentive for additional, more detailed mechanistic studies. Our data also include MST1 in the network of TGFβ and EGF signaling, which may also help improve prediction of responses to drugs currently in use or in clinical trials that target EGF and TGFβ-signaling for treatment of cancer.

2.3.3 Paper 3

PKN1 modulates TGF-β and EGF signaling in HEC-1-A endometrial cancer cell line

Protein kinase N1 (PKN1), also known as PAK1 is a serine/threonine kinase involved in formation of mammary gland tumors and premalignant lesions in animal models, albeit with long latency (Ong *et al*, 2011). PKN1 overexpression has been reported to have an association with prostate, colorectal and aggressive ovarian cancers (Carter *et al*, 2004)(Metzger *et al*, 2003)(Galgano *et al*, 2009). Further studies suggested, PKN1 may also influence the invasive characteristics of breast and gastric cancer cells (Adam *et al*, 2000)(Liu *et al*, 2009).

PKN1 contains three highly conserved regions: (i) a regulatory domain in the N terminal (ii) a catalytically active part located in the C terminal similar to protein kinase C (PKC) (iii) an area referred to as the D region located between the regulatory and catalytic domains (Metzger *et al*, 2003)(Takahashi *et al*, 1998). The N-terminal domain function is crucial for activation of PKN1 (Takahashi *et al*, 1998) by providing the PKN1 activation loop, which is important for serine/threonine kinase activity and the regions required for interaction with other factors participating in the signaling pathway of PKN1, such as Ro GTPases that bind to the hydrophobic region of PKN1 in the N-terminal (Galgano *et al*, 2009). Once activated, PKN1 facilitates downstream signaling actions involved in apoptosis, transformation, cell motility and reorganization of the cytoskeleton. PKN1 also activates various signaling pathways, as well as p38 MAPK, JNK, NF-kB and ERKs (Vadlamudi *et al*, 2000).

Transforming growth factor-β (TGFβ) was found to have a dual role in tumorigenesis. In early-stage cancer, TGFβ has a tumor suppressor role that results in growth inhibition, cell cycle arrest, and apoptosis. Meanwhile, in advance-stage cancer, TGFβ promotes tumorigenesis. The cancer cells may lose responsiveness to TGFβ and may acquire aberrant TGFβ signaling, followed by promotion of survival, proliferation and EMT, as well as increased cell motility and invasiveness (Jakowlew, 2006). TGFβ pathway associate with several signaling pathways including epidermal growth factor (EGF) signaling pathway to modulate its effects (Dunfield & Nachtigal, 2003). EGF is a key regulator of various cellular functions which principally plays a role of a pro-mitogenic molecule in carcinogenesis (Scaltriti & Baselga, 2006). EGF also promotes cell survival, angiogenesis and differentiation. Deregulation of EGF pathways promote tumorigenesis through constitutive activation or overexpression of EGF signaling and is along with a worse prognosis in numerous human malignancies (Lurje & Lenz, 2009).

Signaling cross-talk between different regulators is of key importance for tumorigenesis. The cross-talk may explain modulation of cellular responses to the same regulator by another signaling molecule. Since PKN1 was identified as a potential cross-talk node for TGFβ and EGF signaling, we explored what cellular functions might be affected by PKN1 in cross-talk with TGFβ and EGF. Here we report that PKN1 modulates TGFβ and EGF-dependent regulation of cell

proliferation, migration and invasiveness, and is therefore a component of the network signaling downstream of TGFβ and EGF.

We identified PKN1 as a protein that is deregulated in endometrial cancer (Attarha *et al*, 2013). To study the impact of PKN1 on cells, we transiently expressed wild-type, kinase-negative and constitutively active PKN1 in HEC-1-A endometrial cancer cells. Expression of PKN1 was controlled by immunoblotting with anti-PKN1 antibody. We observed expression of endogenous PKN1 in 6 out 7 tested cell lines. Enhanced expression of PKN1 constructs allowed us to accentuate the impact of PKN1 on cell physiology. Transiently transfected cells were then used in tests described below. Systemic analysis of potential connections between PKN1, TGFβ and EGF showed involvement of a number of potent regulators of cell proliferation and cytoskeleton rearrangement, which prompted us to explore whether PKN1 plays a role in modulation of TGFβ and EGF-dependent regulation of cell proliferation, migration and invasiveness.

We observed that the treatment of cells with TGFβ1 and EGF significantly reduced the proliferation rate of control parental empty vector transfected and wild-type (WT) PKN1 transfected HEC-1-A cells. Transfection of HEC-1-A cells with kinase-inactive (KN) PKN1 and constitutively active (CA) PKN1 resulted in a reduced rate of proliferation of the non-treated cells. We observed that expression of KN PKN1 countered the effects of TGFβ1 and EGF. One unexpected effect was that CA PKN1 countered the effects of EGF and combined TGFβ1 and EGF treatments. The observation with the CA mutant of PKN1 indicates that truncation of PKN1 has a similar impact on the PKN1 contribution as does the KN construct. It also indicates that kinase activity itself is not sufficient to mimic WT PKN1, and PKN1 must be intact to be fully functional. Thus, PKN1 is required for inhibition of cell proliferation through the combined action of TGFβ1 and EGF, since impairment of PKN1 function by blocking the kinase activity or by truncation prevents the inhibitory effect of TGFβ1 and EGF.

To explore whether PKN1 affects cell death, we performed an apoptosis assay. We observed that dual treatment of the empty vector-transfected cells with TGFβ1 and EGF reduced cell apoptosis. Transfection of WT, KN or CA constructs of PKN1 had only a marginal effect on cell death. The only significant, albeit weak, effect was reversal of the effect of combined treatment with TGFβ1 and EGF. We did not observe the presence of apoptotic cells or apoptotic bodies upon visual inspection of transfected and treated cells. Consequently, we observed no strong effects of PKN1 on cell death that could influence cell response to TGFβ1 and EGF in our study.

Since our systemic analysis indicated that PKN1 may affect migration of cells, we performed wound healing (Liang *et al*, 2007) and membrane migration (Penno *et al*, 1997) assays. The wound healing assay explores migration capacities of cells, which are under contact inhibition of proliferation. The membrane migration assay explores proliferating cells in a sparse culture. The molecular mechanisms triggering cell migration in these two tests may differ since the conditions to which the cells are subjected are not equivalent. Despite these differences, the tests may complement each other to allow assessment of migration. The wound healing assay showed that

PKN1 constructs prevented TGFβ1-induced closure. However expression of PKN1 constructs strongly promoted wound closure when cells were treated with both TGFβ1 and EGF.

The membrane migration assay showed that treatment with both TGFβ1 and EGF promoted cell migration. Single treatments and transfections with PKN1 constructs did not have any significant effects. The difference in response pattern between wound healing and membrane migration is that impairment of PKN1 activity in KN and CA mutants did not mimic the WT construct effect seen in the membrane migration assay, compared with the wound healing tests. Despite this difference, our results show that PKN1 may indeed counteract the action of combined treatment of cells with TGFβ1 and EGF.

Invasiveness of cells into a collagen matrix is one of the key mechanisms involved in metastasis. We explored whether PKN1 constructs could affect TGFβ1 and EGF-dependent regulation of cell invasiveness. We observed that treatment of HEC-1-A cells with both TGFβ1 and EGF together increased invasiveness of cells transfected with the KN construct of PKN1, while expression of WT or CA constructs of PKN1 did not have this effect. Expression of KN PKN1 significantly increased cell invasiveness. This stimulatory effect was strong. Thus, PKN1 did modulate invasiveness of cells, with apparently different intracellular mechanisms than those involved in regulation of cell migration.

To explore molecular mechanisms of PKN1 involvement in TGFβ and EGF signaling, we studied expression and phosphorylation of Smad2, Erk1/2, FAK, E-cadherin and vimentin.

Phosphorylation of Smad2 and Erk1/2 reflects activation of signaling downstream of TGFβ and EGF. We observed that expression of WT, KN and CA PKN1 decreased the intensity of Smad2 phosphorylation when treated with TGFβ1. The effect was more significant in CA PKN1 transfected cells. At the same time, phosphorylation of Smad2 decreased in KN and CA PKN1 transfected cells when treated with a combination of TGFβ1 and EGF, or EGF alone, while no change was seen in WT PKN1 transfected cells. We observed that expression of WT, KN and CA PKN1 decreased the intensity of Erk1/2 phosphorylation when treated with TGFβ1. We observed that EGF-dependent Erk1/2 phosphorylation increased in WT, KN and CA PKN1-transfected cells, but not in the vector-transfected cells, indicating that the cells were responsive to EGF when subjected to the enhanced level of PKN1. The effect was most pronounced in KN-transfected cells. Dual treatment of cells with TGFβ1 and EGF had no significant effect on intensity of Erk1/2 phosphorylation in WT and KN transfected cells, while it caused a significant decrease in HEC-1-A CA PKN1 cells.

Expression and activity of focal adhesion kinase (FAK), E-cadherin and vimentin may reflect molecular mechanisms involved in regulation of cell migration and invasiveness. We observed that expression of the WT PKN1 construct increased TGFβ1 and EGF-dependent phosphorylation of FAK, while KN PKN1 expression decreased this effect.

We observed that expression of WT and CA PKN1 predominantly inhibited E-cadherin expression when subjected to either single or dual treatment with TGFβ1 and EGF. The KN construct of PKN1 did not have such an inhibitory effect. Vimentin expression under test conditions was not modulated by treatment with TGFβ1 and/or EGF. The only observed effect involved WT and KN PKN1, which inhibited vimentin levels regardless of treatments. The observations of changes in E-cadherin, vimentin, Smad2 and Erk1/2 expression and phosphorylation indicate that these proteins are indeed affected by PKN1. Our observations justify further studies of the tested proteins in regard to the role of PKN1 in proliferation, migration and invasiveness to gain insight into the underlying molecular mechanisms.

Unraveling the complexity of intracellular signaling cross-talk results in identification of more and more components and interactions between them. Systems biology tools now allow us to identify potential interactions between proteins and genes, which otherwise might remain undetected. Our search for the mechanisms underlying cross-talk between TGFβ and EGF indicate that PKN1 may be a convergence point for these two potent regulatory pathways (Attarha *et al*, 2013). We found that PKN1 is involved as a modulator of cross-talk between TGFβ and EGF in regulation of cell proliferation, migration and invasiveness. The modulating effects of PKN1 were dependent on its kinase activity. The description of cellular responses shows involvement of PKN1 in TGFβ and EGF signaling. The exact molecular mechanisms underlying this cross-talk require further detailed study. Our data indicate that Smad2 and Erk1/2 may be involved in the cross-talk. Smad2 is a direct target of TGFβ receptor type I and Erk1/2 is a convergence target of many different regulators of cell proliferation. It has been shown that Smad2 can be phosphorylated by Erk1/2, and that phosphorylation may inhibit nuclear localization of Smad2 (Kamato *et al*, 2013). Focal adhesion may be regulated by multiple factors, and our results show that PKN1 may interfere with TGFβ- and EGF-dependent phosphorylation of FAK. The effects on FAK phosphorylation are in line with PKN1 modulation of cell invasion and migration. Expression of vimentin and E-cadherin under the explored conditions is also in line with cellular response. It must be noted that the observed correlations indicate involvement of other intracellular components, since the types and amplitudes of changes did not fully overlap with cellular responses. Our systemic analysis and numerous reports by others show that more than 100 molecules may be involved in network signaling by TGFβ and EGF (Katsuno *et al*, 2013b)(Ghosh *et al*, 2011). These molecules are involved in different stages of the signaling cascades, and may include various intracellular processes, such as gene transcription, protein synthesis, protein activities, localization, and metabolic processes. These intracellular processes then exert influence on regulators of cellular responses. Our report includes PKN1 in this network of combined signaling by TGFβ and EGF, which leads to regulation of cell migration, invasiveness and proliferation, and provides direction for further exploration of intracellular mechanisms.

3 General conclusions

The results of studies presented here are expected to contribute to better understanding of the mechanisms underlying human endometrial tumorigenesis, development of novel targets for anticancer treatments, and identification of tumor signatures and prognostic markers for diagnostics and monitoring of endometrial cancer:

- Profiling of individual tumors of EC patients opens up the prospect of treating the specific tumor of an individual patient with tumor-specific cancer drugs. Systemic analysis of individual proteome profiles represented that different proteins may be impacted in the individual endometrial tumors of different patients, but the impact of these proteins on basic cell functions may still be similar. Incorporation of gene expression data sets of profiling of endometrial tumors and our proteome profiling supports the conclusion that individual tumor features are doubtlessly crucial in endometrial tumorigenesis and are not inconsistent individual variations. The potential of PKN1 and MST1 to serve as potential predictive biomarkers of EC, with sufficient sensitivity and specificity to be useful in a clinical setting, is suggested by our data.

- MST1 may act as a negative regulator of the combined action of TGFβ and EGF on cell proliferation, invasiveness and migration of HEC-1-A endometrial cancer cells, while this effect is significantly less pronounced when cells are challenged with each growth factor separately. Monitoring of the intracellular regulatory proteins showed that the MST1 contribution to TGFβ-EGF cross-talk may involve focal adhesion kinase and E-cadherin, but not activation of Smad2. These observations underscore the importance of exploring combination treatments.

- Analysis of PKN1 involvement in the cellular regulatory processes indicated its role as a point of convergence for TGF-β and EGF initiated signaling which has inhibitory effect on stimulation of cell migration and that its kinase activity was required for the inhibitory effect on cell proliferation and invasiveness. Monitoring of the intracellular regulatory proteins showed that phosphorylation of Smad2, FAK and Erk1/2 correlated with cellular response to TGFβ1 and EGF. These observations pave the way for further exploration of intracellular mechanisms.

Despite identification of numerous EC-associated proteins, our knowledge of proteomics analysis of endometrial carcinoma is poorly developed. Lack of commonly used biomarkers in routine clinical practice for detection of early stage or aggressive EC promoted us to perform such analysis which expected to provide abundant EC-associated proteins for building of proteome signatures for daily clinical use.

4 ACKNOWLEDGEMENTS

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