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Molecular profiling of signal transduction pathways regulating the E-cadherin repressor Snail in human gynaecological cancers

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Poster: Characterisation of signal transduction pathways regulating Snail in primary human endometrial carcinoma

Contents

1 Introduction

1.1 Endometrial carcinoma

In developed countries endometrial cancer is the most common cancer of the female genital tract and the fourth most common malignancy in women after breast, lung and colorectal cancers (Amant et al., 2005; Jemal et al., 2005; Parkin et al., 2005; Parkin et al., 1999). Endometrial carcinomas can be divided on clinical and prognostic grounds into two clinicopathological subtypes (Bokhman, 1983). Type I tumours are oestrogen dependent endometrioid endometrial carcinomas and comprise about 80 % of sporadic endometrial cancers. These tumours occur in pre- and postmenopausal women, are low grade with endometrioid morphology and have a good prognosis. Type II tumours are non-oestrogen dependent endometrial carcinomas with a clear-cell or papillary serous morphology. These tumours occur mainly in postmenopausal women, are high grade tumours and have a poor prognosis (Bokhman, 1983; Hecht & Mutter, 2006; Lax, 2004; Matias-Guiu et al., 2001; Ryan et al., 2005; Sorosky, 2008).

1.2 Ovarian carcinoma

Ovarian carcinoma is the second most common cancer after endometrial cancer of the female genital tract and the fourth leading cause of cancer related death among women in the developed world (Ahmed et al., 2007; Parkin et al., 2005; Whittemore, 1994). 5-year survival is good, if diagnosed at an early stage when the cancer is confined to the ovary. However, almost 70 % of women are diagnosed at an advanced stage, at which point 5-year survival is 30 % (Corney & Nikitin, 2008). Because of lack of symptoms in early stages of the disease ovarian carcinoma is often not diagnosed until in late stages and not before metastatic setting of the tumour (Goff et al., 2000; Sheehan et al., 2005), when the chance of cure is pure. Two thirds of patients are diagnosed with stage III or IV (Davidson et al., 2000). Ovarian cancer has the highest mortality rate of any gynaecological malignancy (Bourguignon et al., 2007; Davidson et al., 2000).

1.3 Grading and staging of tumours

The tumour grade (**Table 1-1**) mirrors the morphology and proliferative capacity of primary tumours. The stage of a tumour describes the expansion of the tumour locally or at a distance from the primary site. According to the International Union against Cancer (UICC), staging, in contrast to grading, is based on clinical characteristics at the time of diagnosis, including the size of the tumour, the status of lymph nodes near the tumour and the presence of metastasis (Thiery, 2002). The staging of endometrial and ovarian carcinoma is given in **Table 1-2** and **Table 1-3**.

Table 1-1 Grading of tumours

GX: Grade cannot be assessed

G1: Well differentiated (Low grade)

G2: Moderately differentiated (Intermediate grade)

G3: Poorly differentiated (High grade)

Table 1-2 UICC Staging of primary carcinoma of the endometrium. Taken from: The AJCC Cancer staging manual. (American Joint Commitee on Cancer, 2002; Wittekind et al., 2003)

UICC stage	Definition
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
Tis	Carcinoma in situ
1	**Tumour confined to corpus uteri**
1a	Tumour limited to endometrium
1b	Tumour invades up to or less than one-half of the myometrium
1c	Tumour invades to more than one-half of the myometrium
2	**Tumour invades cervix but does not extend beyond uterus**
2a	Endocervical glandular involvement only
2b	Cervical stromal invasion
3	**Local and/or regional spread**
3a	Tumour involves uterine serosa and/or adnexa (direct extension of metastasis) and/or cancer cells in ascites of peritoneal washings
3b	Vaginal involvement (direct extension or metastasis)
3c	Metastasis to the pelvic and/or para-aortic lymph nodes
4a	Tumour invades bladder mucosa and/or bowel mucosa
4b	Distant metastasis (Excluding metastasis to vagina, pelvic serosa, or adnexa. Including metastasis to intra-abdominal lymph nodes other than para-aortic, and/or inguinal lymph nodes)

Table 1-3 FIGO Staging for primary carcinoma of the ovary. Taken from: The AJCC Cancer staging manual. (American Joint Commitee on Cancer, 2002; Wittekind et al., 2003)

FIGO stage	Definition
1	**Growth limited to the ovaries**
1a	Growth limited to one ovary; no ascites containing malignant cells. No tumour on the external surface; capsule intact.
1b	Growth limited to both ovaries; no ascites containing malignant cells. No tumour on the external surfaces; capsules intact.
1c	Tumour either stage 1a or 1b but with tumour on the surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
2	**Growth involving one or both ovaries with pelvic extension**
2a	Extension and/or metastases to the uterus and/or tubes.
2b	Extension to other pelvic tissues.
2c	Tumour either stage 2a or 2b, but with tumour on the surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
3	**Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals stage 3. Tumour is limited to the true pelvis, but with histological proven malignant extension to small bowel or omentum.**
3a	Tumour grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
3b	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative.
3c	Abdominal implants > 2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
4	**Growth involving one or both ovaries with distant metastasis. If pleural effusion is present there must be positive cytological test results to allot a case to stage 4. Parenchymal liver metastasis equals stage 4.**

1.4 Epithelial-mesenchymal transition in tumour progression

Epithelial-mesenchymal transition (EMT) is a fundamental process governing morphogenesis in multicellular organisms. This process is reactivated in wound healing and a variety of diseases, including fibrosis and progression of carcinoma (Thiery, 2003; Thiery & Sleeman, 2006).

Most human solid tumours are carcinomas that originate from epithelial cell types. The tumour cells must lose cell-cell adhesion and acquire motility to break away from neighbouring cells to invade bordering cell layers. The cellular motility shares many similarities with the cellular migration and tissue rearrangement during various developmental events. Tumours are often viewed as corrupt forms of normal developmental processes. Genes that are important in embryonic development are frequently found to play a role in cancer progression. Conversely, genes discovered for their oncogenic role are often found to be key players in embryogenesis (Kang & Massague, 2004).

Epithelial and mesenchymal cells represent two of the main cell types in mammals. Epithelial cells are characterized by (1) cohesive interactions between cells, alleviating formation of cell layers, (2) existence of three membrane domains: apical, lateral and basal, (3) tight junctions between apical and lateral domains, (4) apicobasal polarized allocation of the various organelles and cytoskeleton components, (5) lack of motility and (6) a cobblestone-like morphology. Mesenchymal cells are characterized by (1) no interactions among cells, (2) no apical and lateral membranes, (3) no apicobasal polarization, (4) motility and invasive behaviour and (5) spindle-shaped morphology (Larue & Bellacosa, 2005).

EMT is a highly conserved and fundamental process that governs morphogenesis in multicellular organisms. But also the reverse process mesenchymal-epithelial transition (MET) is known from developmental processes, it has been described that mesenchymal cells can also participate in the formation of epithelial organs, e. g. heart and kidney, through MET (Thiery, 2002).

EMT and MET in carcinogenesis are defined by four major changes in cellular phenotype (Boyer & Thiery, 1993; D'Souza-Schorey, 2005; Hugo et al., 2007; Kang & Massague, 2004; Thiery, 2002; Yang & Weinberg, 2008) (**Figure 1.1**):

1) Genetic alterations lead to morphological changes from a cobblestone-like monolayer of epithelial cells with an apical-basal polarity to dispersed, spindle-shaped mesenchymal cells with migratory protrusions, lost epithelial polarity and enhanced proliferation potential.
2) Changes of expression of differentiation markers from cell-cell junction proteins and Cytokeratin intermediate filaments, e.g. E-cadherin and Cytokeratin 18, to Vimentin filaments and Fibronectin.

3) Functional changes associated with the dissemination of the basement membrane, rearrangement of the actin cytoskeleton and conversion of stationary cells to motile cells that can invade through the extra cellular matrix into blood or lymph vessels.
4) Once reached distant organs the tumour cells can extravasate and either remain solitary as micrometastasis or they recapitulate the pathology of their corresponding primary tumour by undergoing mesenchymal-epithelial transition.

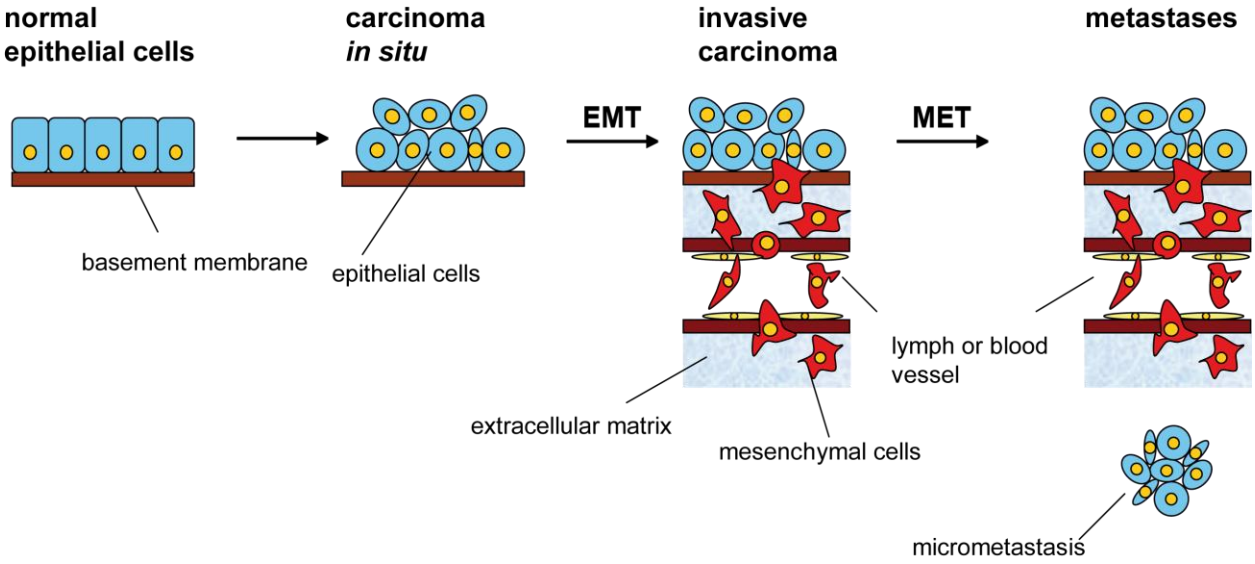

Figure 1.1 EMT and MET in carcinoma progression. Modified from Thiery 2002.

1.4.1 Types of cell-cell contacts in epithelial tissues

In epithelial tissues, cells are connected to each other by adherence junctions, arranged by cadherins, tight junctions, connected by occludin and claudin and desmosomes, mediated by desmoplakin. Adherence junctions and desmosomes are members of the anchoring junctions, responsible for strong cell-cell connections mediated by transmembrane proteins connected to actin (adherence junctions) and intermediate (desmosomes) filaments respectively. Tight junctions belong to the group of occluding junctions, they tight close the gap between two cells (Alberts et al., 2002; Hugo et al., 2007).

1.4.2 E-cadherin

Cohesive sheets of epithelial cells are a fundamental feature of multicellular organisms and are largely a product of the varied functions of adherence junctions (Perez-Moreno et al., 2003). Adherence junctions are responsible for epithelial cell-cell adhesion and polarity. The disassembly of adherence junctions results in a loss of the polarized, poorly motile, epithelial phenotype and the acquisition of a migratory or mesenchyme-like phenotype. Cadherins are the main proteins of adherence junctions and are responsible for homotypic cell-cell adhesion. Cadherin molecules are integral membrane glycoproteins with a single transmembrane domain (D'Souza-Schorey, 2005). Loss of E-cadherin is a hallmark of EMT and E-cadherin is therefore emerging as one of the caretakers of the epithelial phenotype (Thiery, 2002). The extra cellular domain of E-cadherin consists of five cadherin domains which are responsible for the homophilic interaction of these molecules. Each of these parts contains two Ca^{2+} binding sites, which are contemplated to play key roles in Ca^{2+}-protein and protein-protein interactions. The association of Ca^{2+} ions with a linker region connecting two of the extra cellular domains induces the conformational changes necessary for the extra cellular domain of cadherins to mediate their adhesive interactions. E-cadherin mainly forms homophilic interactions. The cytoplasmatic domain of the cadherins is connected via catenins to the actin cytoskeleton (**Figure 1.2**). Although the extra cellular domain is sufficient to arrange cell-cell contacts, the cytoplasmatic domain of cadherins is essential to enhance the force of this interaction and for cellular signalling (Hirohashi, 1998; Peinado et al., 2004).

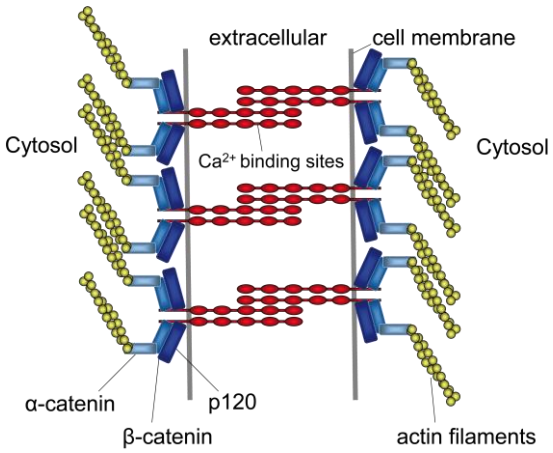

Figure 1.2 Schematic structure of adherence junctions mediated by homophilic cell-to-cell interactions. E-cadherin is the most prominent protein of adherence junctions of epithelia.

Cell adherence junctions are mechanically stable. When E-cadherin is sufficiently active, cancer cells cannot disrupt their mutual connections, but suppression of E-cadherin function may trigger the release of cancer cells from primary tumours, so called metastases (Hirohashi, 1998). So, disruption of cell-cell contacts is one of the key events in tumour progression (Peinado et al., 2004) and leads to the conclusion that E-cadherin may act as a tumour suppressor (Birchmeier & Behrens, 1994). E-cadherin expression can be modified by various mechanisms like gene mutations (Becker et al., 1994; Becker & Hofler, 1995; Berx et al., 1998), promoter hypermethylation (Grady et al., 2000; Machado et al., 2001), post-translational modification (Rashid et al., 2001) and transcriptional repression (Batlle et al., 2000; Cano et al., 2000; Hemavathy et al., 2000).

1.4.2.1 Transcriptional regulation of E-cadherin

The human E-cadherin promoter is a TATA-less promoter containing several regulatory elements, including a CCAAT box, a GC-rich region and three E-boxes. The CCAAT box and the GC rich-region are required for basal E-cadherin expression, the E-boxes are responsible for E-cadherin repression (**Figure 1.3**) (Batlle et al., 2000; Peinado et al., 2004).

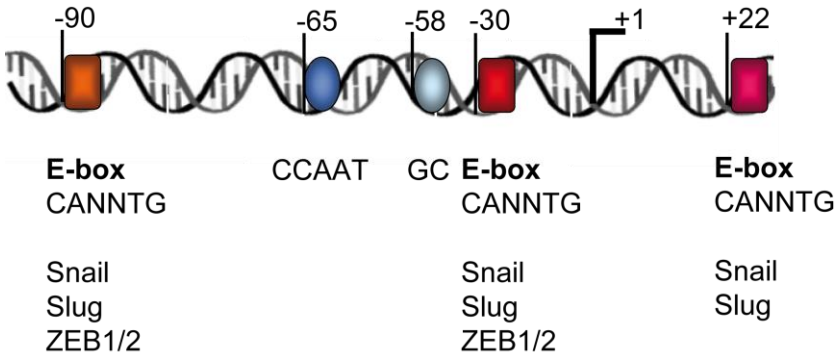

Figure 1.3 Schematic representation of the human E-cadherin promoter. Modified from Battle et al., 2000 and Peinado et al., 2004.

Several transcription factors that strongly repress E-cadherin, such as members of the Snail (Snail, Slug), ZEB (ZEB1, ZEB2) and basic-helix-loop-helix (Twist1, Twist2) families, are thought to be involved in tumour progression (Peinado et al., 2007) (**Figure 1.4**).

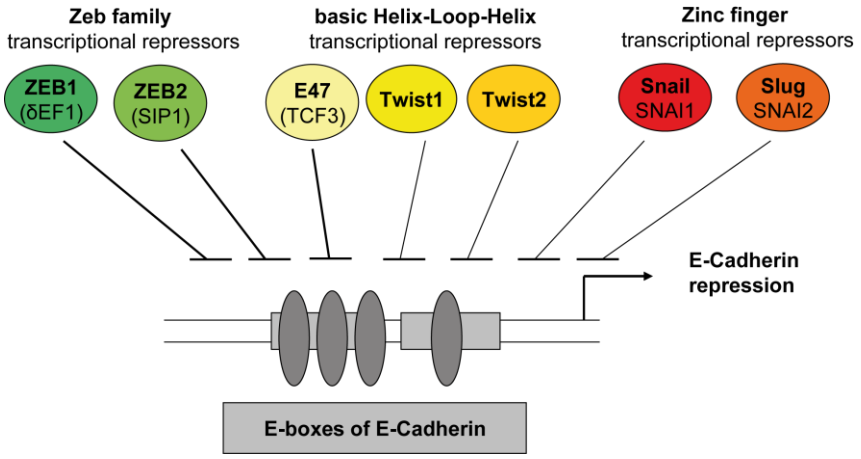

Figure 1.4 Transcriptional repressors of E-cadherin. Transcription factors of the zinc finger, basic Helix-Loop-Helix and the ZEB family target the E-boxes in the E-cadherin promoter. Modified from: Huber et al., 2005 and Peinado et al., 2007)

Snail factors

Three members of the Snail family have been described in vertebrates: SNAI1 (Snail), SNAI2 (Slug) and SNAI3 (Barrallo-Gimeno & Nieto, 2005). Members of the Snail family are zinc-finger transcription factors that share a common organization (**Figure 1.5**): The C-terminal DNA-binding domain (amino acids 152 to 264) contains four zinc fingers belonging to the C_2H_2 subclass. This domain is conserved in other vertebrate and invertebrate homologues of the protein and has the highest affinity for oligonucleotides containing a 5'-CACCTG-3' sequence (Dominguez et al., 2003). Yamasaki et al. showed that the zinc finger domain of Snail plays a role in nuclear localisation, which is mediated by importin β (Ko et al., 2007; Yamasaki et al., 2005). In the central region of Snail two functional domains have been found: a regulatory domain containing a nuclear export signal (NES) and a destruction box domain (Peinado et al., 2007; Zhou et al., 2004). Control of nuclear localisation of specific proteins is an important mechanism in the regulation of many signal transduction pathways. Transcription factors cannot function until they are translocated to the nucleus in response to specific signals. The N-terminal domain differs much more among the various Snail homologues. In mammals, a short sequence in the amino terminus, called SNAG domain, is essential for repression (Dominguez et al., 2003).

The phosphorylation of the Serin-Proline-rich region in the NES domain and potential modification of adjacent lysine residues has been implicated in the sub cellular localisation of Snail protein stability and repressor activity (Dominguez et al., 2003;

Peinado et al., 2005; Zhou et al., 2004). Yamasaki et al. revealed that all four zinc fingers of Snail are necessary for efficient nuclear localisation (Yamasaki et al., 2005).

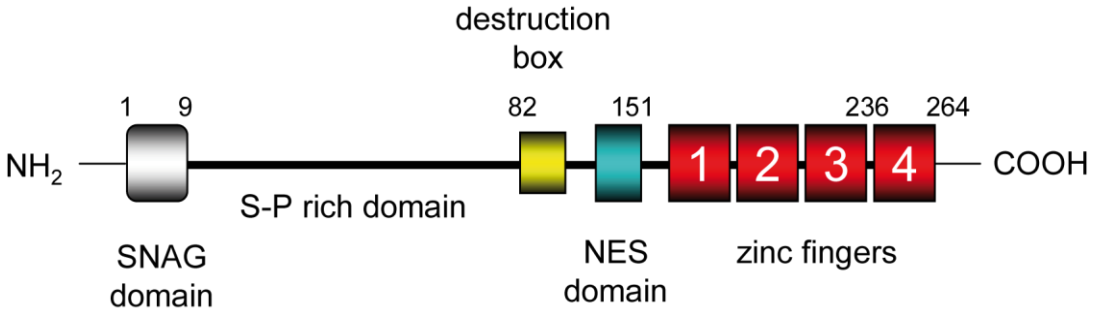

Figure 1.5 Diagram of the main structural domains found in human Snail. Modified from Dominguez et al., 2003 and Peinado et al., 2007.

Basic-helix-loop-helix factors

The basic common structure of basic-helix-loop-helix factors (bHLH) consists of two parallel amphipathic α-helices joined by a loop required for dimerisation. They bind to DNA using a consensus E-box (CANNTG) site as homo- or heterodimers. E47, Twist1 and Twist2 are the most important basic-helix-loop-helix factors playing a role in EMT (Peinado et al., 2007).

ZEB family factors

The ZEB family of transcription factors contains two members, ZEB1 (also known as δEF1) and ZEB2 (also known as SMAD interacting protein 1 (SIP1)). ZEB factors interact with DNA through simultaneous binding of the two zinc finger domains to high-affinity binding sites composed of E-boxes (CACCTG) (Peinado et al., 2007).

Current evidences indicate that silencing of E-cadherin transcription requires several repressors that interact with specific E-boxes in the promoter. Peinado et al. proposed a model for participation of the different E-cadherin repressors in tumour progression (**Figure 1.6**). Thereby, the transient expressions of Snail or SIP1 initiate the invasion process. Other repressors, like Slug, E47 and δEF1 will be involved in the maintenance of the invasive phenotype. The authors also suggest specific participation of each repressor in distinct types of carcinoma (Peinado et al., 2004).

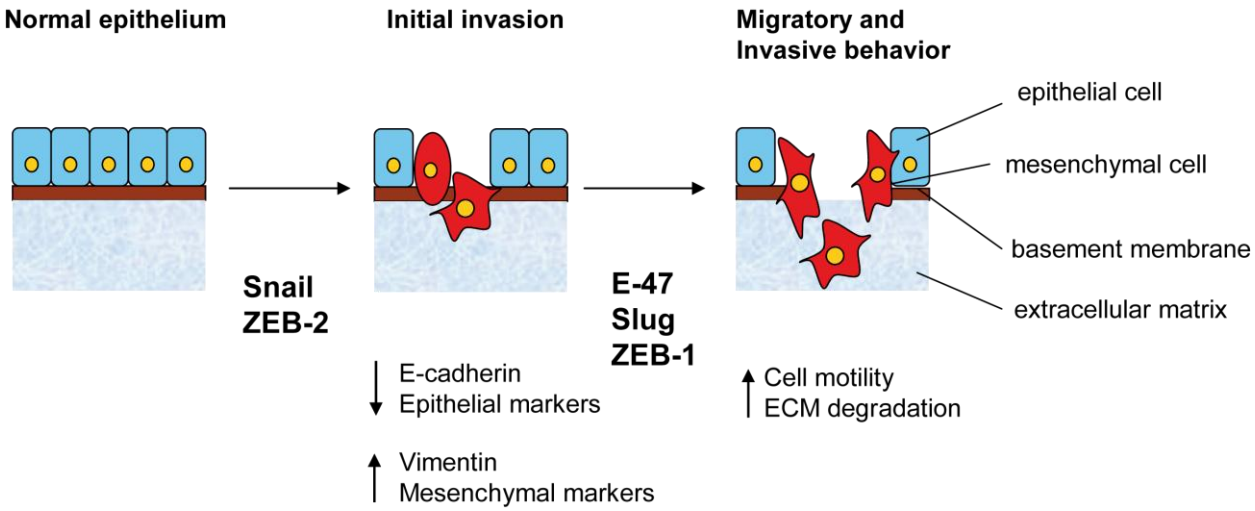

Figure 1.6 Model of the participation of different E-cadherin repressors during tumour progression. Modified from Peinado et al., 2004

1.4.3 Snail expression in tumours of the endometrium and ovary

It has recently been reported that Snail may play a major role in hormone dependent carcinomas, including endometrial and ovarian cancer, than in cancers of the gastrointestinal tract, including gastric and colon cancer (Becker et al., 2007a).

Blechschmidt et al. found an inverse correlation of Snail and oestrogen receptor α expression in primary endometrial cancers. In metastases of endometrial carcinoma Snail correlated with a higher tumour grade and reduced E-cadherin expression (Blechschmidt et al., 2007).

Blechschmidt et al. not only analysed Snail expression in endometrial cancer, but also in ovarian carcinomas. They found a significant correlation between Snail expression in primary tumours and their corresponding metastases. Additionally, patients with reduced E-cadherin and increased Snail expression in primary tumours and metastases had significantly higher risk of death (Blechschmidt et al., 2008).

1.4.4 Signalling pathways regulating Snail in human tumours

Snail controls cell motility and is involved in cancer progression but Snail has also other functions associated with its role as trigger of EMT. Additionally with the induction of EMT, Snail expression can confer survival properties to the cells (Hemavathy et al., 2000; Leroy & Mostov, 2007; Valdes et al., 2002; Vega et al., 2004).

1.4.4.1 Transforming growth factor β signalling

Transforming growth factor β (TGFβ) plays a bimodal role in many cancers, ranging from inhibitory in early stages to tumour enhancing in advanced stages of cancer (Van Overveld, 2007). Moustakas and Heldin reported that in all *in vitro* models, analysed so far, TGFβ down-regulates various epithelial proteins, including E-cadherin, ZO-1 and keratins and up-regulates mesenchymal proteins, such as Fibronectin and Vimentin (Moustakas & Heldin, 2007). It has been shown that Snail functions as a molecular mediator of TGFβ1-regulated MMP-9 expression in oral squamous cell carcinoma cells at the transcriptional level, thereby contributing to oral cancer progression (Sun et al., 2008). In Madin-Darby canine kidney (MDCK) cells Peinado et al. showed that TGFβ1 induces the activity of the Snail promoter (Peinado et al., 2003). Gotzmann et al. described that TGF-β1, -β2 and –β3 are specific for induction of hepatocellular EMT during tumour progression (Gotzmann et al., 2006).

1.4.4.2 Wnt/β-Catenin signalling

β-Catenin interacts with E-cadherin at adherence and tight junctions to maintain the epithelial phenotype. The adhesive function of E-cadherin is dependent on its binding to the cytoplasmatic catenins, which connect E-cadherin to the cytoskeleton (Yook et al., 2005). In response to exogenous signals, β-catenin translocates from the cell membrane into the cytoplasm. There it is either degraded, or translocated into the nucleus, where it can regulate gene transcription and induce EMT (Acloque et al., 2008).

1.4.4.3 Oestrogen receptor α signalling

Oestrogen receptor α (ERα) is a key regulator of proliferation and differentiation in epithelia of the female reproduction system. It induces changes in gene expression through direct gene activation and also through the biological functions of target loci. Fujita et al. demonstrated that the absence of ERα leads to aberrant expression of Snail via expression of MTA-3 and transfection of ERα leads to repression of Snail expression in breast cancer (Fujita et al., 2003). In contrast to these findings Park et al. reported up-regulation of Snail after 17β-estradiol (E2) stimulation in human ovarian cancer cell lines (Park et al., 2008).

1.4.4.4 Hypoxia

Highly aggressive and fast growing tumours outgrow their blood supply, leaving the cells starved of oxygen, a condition known as hypoxia (Bernards, 2003). Imai et al.

suggests that hypoxia contributes to tumour progression and to epithelial-mesenchymal transition by inactivating E-cadherin via Snail in ovarian carcinomas (Imai et al., 2003). But the exact mechanism still remains unclear.

1.4.4.5 EGFR family

The human epidermal growth factor (HER) gene family (HER1-HER4) encodes structurally similar transmembrane proteins (EGFR (erbB1, HER1), HER2 (erbB2), HER3 (erbB3) and HER4 (erbB4)) with intrinsic tyrosine kinase activity (Hackel et al., 1999; Krawczyk et al., 2003). The HER receptors are localised on the cell surface and may be activated by binding of specific ligands, e.g. epidermal growth factor (EGF) and TGF α. Upon activation, EGF receptors undergo dimerization, autophosphorylation and stimulate cell proliferation (Alberts et al., 2002). The erbB2 receptor has no known direct ligand and may be activated constitutively or become activated after heterodimerization with an other family member e.g. EGFR. Autophosphorylation leads to downstream activation. Activated EGFR invokes several downstream signalling cascades, including mitogen activated kinases (MAPK), signal transducers and activators of transcription (STAT) and protein kinase B (AKT) pathways, in several cell types (Hackel et al., 1999; Kothmaier et al., 2008; Lu et al., 2003; Yamane et al., 2007).

EGFR signalling is frequently deregulated in epithelial tumours and it has been shown, that EGF treatment of tumour cells leads to down-regulation of E-cadherin, up-regulation of Vimentin and enhanced invasive behaviour of tumour cells (Barr et al., 2008; Lee et al., 2008; Lu et al., 2003). Lu et al. also reported that EGF-induced up-regulation of Snail mRNA is necessary for down-regulation of E-cadherin in 293 cells (Lu et al., 2003). In cervical cancer cells it was shown that EGFR signalling inactivates glycogen synthase kinase 3β (GSK-3β). Therefore Snail accumulates in the nucleus und represses epithelial markers (Lee et al., 2008) (for more details see **chapter 1.4.4.9**).

1.4.4.6 PI3K, AKT and PTEN

Phosphatidylinositol 3-kinase (PI3K) catalyzes the production of phosphatidylinositol-3,4,5-triphosphate (PIP_3) from the precursors phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP_2). The increase in phosphoinositides leads to the recruitment of AKT to the cell membrane, phosphorylation and thereby activation of AKT. The PI3K/AKT pathway normally promotes cell survival and cell growth (Alberts et al., 2002). The phosphatase

and tensin homologue deleted on chromosome ten (PTEN) dephosphorylates phosphatidylinositol 3,4,5-triphosphate and acts as a negative regulator of the PI3K/AKT pathway, resulting in reduced cell migration, cell proliferation and increased apoptosis (Alberts et al., 2002; Lodish et al., 2001; Mellinghoff et al., 2007). The tumour suppressor PTEN is the most commonly altered component of the PI3K pathway in human malignancies. Mutations occur in both heritable and sporadic settings (Chow & Baker, 2006).

PI3K/AKT is activated in many human carcinomas, and AKT-driven EMT may confer the motility required for tissue invasion and metastasis (Grille et al., 2003). PTEN is frequently deleted in human tumours, leading to enhanced cell-cycle progression and reduced apoptosis (Larue & Bellacosa, 2005; Lodish et al., 2001). Grille et al. reported that active AKT down-regulates the expression of the E-cadherin gene transcriptionally by inducing Snail expression (Grille et al., 2003).

1.4.4.7 Nuclear Factor κB

The nuclear factor κB (NF-κB) signal transduction pathway plays important roles in the maintenance of certain cell phenotypes through regulating the expression of a large number of genes, including Snail and Vimentin (Saegusa et al., 2007). The NF-κB family is comprised of five proteins in mammals: p65/Rel-A, c-Rel, Rel-B, NF-κB1 (p105/p50), and NF-κB2 (p100/p52), each containing a Rel homology domain that mediates DNA binding and dimerization (Saegusa et al., 2007).

Activation of transcription factor NF-κB occurs in many tumours, and studies have shown that NF-κB can promote cell proliferation and oncogenesis, possibly by protecting cells from apoptosis. In mammary carcinogenesis it was shown that NF-κB was essential for induction and maintenance of EMT and metastasis (Huber et al., 2004a; Huber et al., 2004b). Barberà et al. described for the first time that NF-κB/p65 stimulated Snail transcription (Barbera et al., 2004). Julien suggested that Snail activation and consequent repression of E-cadherin may depend on AKT-mediated NF-κB activation, and that NF-κB induces Snail expression (Julien et al., 2007).

1.4.4.8 Mitogen-activated protein kinases

The mitogen-activated protein kinases (MAPK) are a family of serine/threonine kinases that play an important role in signal transduction. They include the extra cellular signal-regulated protein kinases (ERK1, ERK2, also known as p44MAPK and p42MAPK), c-Jun N-terminal kinases (JNK1, JNK2, JNK3), p38 MAPKs (p38α, p38β, p38γ, p38δ) and

ERK5 (Turjanski et al., 2007). Once activated e.g. by growth factors, they phosphorylate numerous proteins, including transcription factors, cytoskeletal proteins, kinases and other enzymes, and influence gene expression, metabolism, cell division, cell morphology and cell survival (Qi & Elion, 2005).

The ERK1/2 pathways are activated in response to growth factors and hormones whereas p38 MAPK pathways are activated in response to cellular stress. MAPKs are involved in many physiological processes, including cell proliferation, differentiation and survival. The activation of ERK1/2 induces proliferative signals that may contribute to normal and cancerous cell growth (Owens & Keyse, 2007; Turjanski et al., 2007). Hudson identified ERK1/2 and p38 MAPK pathways as mediators of Snail and Slug up-regulation after UVR exposure in epidermal keratinocytes (Hudson et al., 2007).

1.4.4.9 Glycogen synthase kinase 3β

Glycogen synthase kinase 3β (GSK-3β) is a serine/threonine kinase that phosphorylates numerous substrates including transcription factors, structural proteins and signalling proteins (Doble & Woodgett, 2007).

GSK-3β regulates Snail by two independent mechanisms: transcriptional repression (Bachelder et al., 2005) and phosphorylation (Huber et al., 2005; Zhou et al., 2004).

GSK-3β binds to and phosphorylates Snail at two consensus motifs to dually regulate its function. Phosphorylation of the first motif regulates its β-Trcp-mediated ubiquitination and causes Snail to undergo degradation. Whereas phosphorylation of the second motif, controls its sub cellular localisation and causes Snail to localise in the cytoplasm, where it cannot function as a transcriptional repressor. Snail protein is highly unstable, with a half-life of about 25 minutes. According to this model, in the first step GSK-3β binds to and phosphorylates Snail and induces its nuclear export and once in the cytoplasm, in the second step GSK-3β phosphorylates Snail on a second motif which results in the association of Snail with β-Trcp and its subsequent degradation (Peinado et al., 2005; Schlessinger & Hall, 2004; Wang & Hung, 2005; Yook et al., 2005; Zhou et al., 2004). Mutations in the Snail gene that prevent GSK-3β-mediated phosphorylation result in a stabilized form of Snail that localises in the nucleus and induces EMT (Thiery & Sleeman, 2006). GSK-3β can be inactivated by EGF and insulin-like growth factor through phosphorylation of Ser-9 by the PI3K/AKT and ERK pathways (Schlessinger & Hall, 2004; Wang & Hung, 2005), what results in reduced Snail degradation.

1.4.4.10 p21 activated kinase 1

p21 activated kinases (PAK), an evolutionarily conserved family of serine/threonine kinases, are essential for a variety of cellular functions, including cell morphogenesis, cell motility, cell survival, angiogenesis and mitosis (Jaffer & Chernoff, 2002; Wang et al., 2006). PAK1 over-expression has been reported in human ovarian and breast cancer (Balasenthil et al., 2004; Holm et al., 2006). PAK1 is activated by a number of growth factors, e.g. heregulin and epidermal growth factor. Yang et al. described that PAK1 phosphorylation of Snail on Serine 246 promotes Snail's nuclear accumulation and consequently its repressor activity in the nucleus in human breast cancer cell lines. Furthermore, PAK1 enhanced the ability of Snail to repress E-cadherin and Occludin promoter activities (Yang et al., 2005). Given the functional importance of the monopartite nuclear localisation motif proximal to zinc finger, it is therefore suggested that phosphorylation on zinc finger by PAK1 adjacent to these motifs may play a role in the nuclear transport of Snail (Ko et al., 2007).

1.4.4.11 LIV-1

LIV-1 belongs to the ZIP (Zrt- and Irt-like proteins) family of zinc transporters and is located in the plasma membrane. Zinc plays an important role in controlling cellular processes of the cell, such as growth, development and differentiation (Taylor & Nicholson, 2003). Zinc transporters play an important role in maintaining intracellular zinc homeostasis, aberration of which could lead to diseases such as cancer (Taylor et al., 2003). Yamashita et al. reported a significant association between STAT3, LIV-1 and Snail. They identified LIV-1 as a downstream target of STAT3 that is essential for the nuclear localisation of zinc finger protein Snail in gastrula organizer cells during zebra fish embryogenesis (Taylor et al., 2004; Yamashita et al., 2004). Zhao et al. described the first time that LIV-1 is over-expressed in cervical cancer and is involved in invasion of cervical cancer cells through targeting ERK1/2-mediated Snail and Slug expression (Zhao et al., 2007).

1.5 Extraction of full-length proteins from formalin fixed and paraffin embedded tissues

Cancer is both a genomic and proteomic disease. During cancer progression genetic defects lead to alteration of signal transduction pathways, leading to enhanced cell

growth, proliferation, survival, invasion and reduced apoptosis of cancer cells. Protein analysis provides fundamental information about the functional state of signalling pathways (Espina et al., 2003; Liotta et al., 2003).

In most hospitals around the world formalin fixed and paraffin embedded tissues (FFPE) are used for histopathological diagnosis. Formalin causes cross-links between macromolecules and keeps the tissue in an excellent condition (Becker et al., 2007b). DNA and RNA extraction from those tissues have been successful applied and are already in use in routine diagnostic in hospitals (Specht et al., 2001). But it is long known that the concentration of a mRNA within a cell does not necessarily correlate with abundances of that protein (Mitchell, 2002). However proteins are the action molecules or the verbs of a cell (Espina et al., 2004). Therefore analysis of protein networks in tumour tissues is very important for diagnosis and therapy. Until recently immunohistochemistry (IHC) was the only method to analyse protein expression in formalin fixed and paraffin embedded tissues. The advantage of IHC is, that the localisation of the protein inside the cell can be determined, but it is not suitable for the analysis of subtle quantitative changes in multiple classes of proteins taking place simultaneously within a cell or tissue (Paweletz et al., 2001). Although it was long believed that formalin caused cross-linking of proteins is irreversible, several groups described successful protein extraction from formalin fixed and paraffin embedded tissues in the last few years (Becker et al., 2007b; Chu et al., 2005; Ikeda et al., 1998).

1.6 Protein lysate microarrays

Proteins are, in contrast to DNA and RNA, highly complex molecules and may exist in a broad dynamic range, up to a factor 10^{10} in any cell (Espina et al., 2003). DNA is composed of four nucleotides and is a molecule with a defined hydrophilic backbone and structure. The human proteome is much more complex than the genome. Proteins consist of 20 amino acids able to form tertiary and quaternary structures. Proteins may be acidic, basic, hydrophobic or hydrophilic and may be post-translationally modified, e.g. phosphorylated or glycosylated (Espina et al., 2004; Gulmann et al., 2006).

Unlike for DNA and RNA, amplification methods, like PCR, do not exist for proteins and the sample volume of clinical materials like tumour tissues or biopsies is limited. Consequently very sensitive (at least femtomolar range) methods with a high linear range are needed to quantify protein expression in tissues (Liotta et al., 2003).

In the simplest sense, reverse phase protein microarrays are immobilized protein spots followed by quantitative immunochemical detection (Espina et al., 2003; Nishizuka & Spurrier, 2008; Wulfkuhle et al., 2006) (**Figure 1.7**). This array format allows multiple samples to be analysed for expression of one protein under the same experimental conditions (Espina et al., 2003; Liotta et al., 2003).

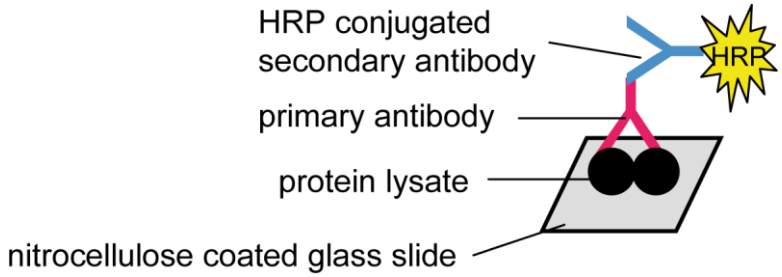

Figure 1.7 Protein lysate microarray. Protein lysates are immobilized on nitrocellulose coated glass slides and detected by specific antibodies. For detection chemiluminescence can be used.

In reverse phase microarrays the whole repertoire of patient proteins that represent the state of an individual cell population, undergoing disease progression, can be analysed (Paweletz et al., 2001). With this array format a map of known signalling proteins that generally reflect the state of information flow through protein networks in individual specimens can be generated. Furthermore, protein microarrays that examine protein-protein recognition events, e.g. phosphorylation, can be used to profile cellular signalling pathways in a way not possible with gene arrays (Paweletz et al., 2001; Wulfkuhle et al., 2006). Paweletz et al. were the first who used reverse phase protein microarrays to study signal transduction pathways in human tissues. They achieved high sensitivity, precision and linearity for their microarrays and were able to quantify the phosphorylated status of signal proteins in human tissue cell subpopulations (Paweletz et al., 2001). Phosphoprotein driven cellular signalling events represent most of the new molecular targets for cancer treatment. Protein lysate microarrays have the potential for identification and characterisation of interconnecting protein pathways and networks in tissues (Gulmann et al., 2006; Liotta et al., 2003; Paweletz et al., 2001). Thus this technology holds the promise of becoming one of the most important tools for the analysis of cell and tissue physiology as a set of linked networks. Linearity of protein detection is ensured by using a dilution curve for each lysate analysed. A limitation of the current protein microarray methodology was that it could not be used with routinely

processed clinical samples, e.g. formalin-fixed tissues, most likely due to protein crosslinks emerging from formalin fixation. As described in **chapter 1.5** it was recently shown that protein extraction from formalin fixed and paraffin embedded tissues is possible (Becker et al., 2007b; Chu et al., 2005; Ikeda et al., 1998). Therefore mapping of signal transduction pathways activated in the tumour is encouraging for diagnosis and therapeutic selection, in addition to conventional histology (Wulfkuhle et al., 2006; Wulfkuhle et al., 2008).

2 Aim of the thesis

Alterations of cell adhesion molecules are often seen in human carcinomas, including endometrial and ovarian cancer, resulting in enhanced invasion and metastasis. The epithelial-mesenchymal transition regulator Snail directly inhibits the cell adhesion molecule E-cadherin, which is part of adherence junctions. In previous studies it was shown that Snail plays a role in tumour progression in endometrial carcinoma and is associated with lower overall survival of ovarian cancer patients.

The regulation of the transcription factor Snail in human cancers, however, is not known in detail. Many signalling pathways (MAPK, AKT, GSK-3β, EGFR, ERα) have been described to play a role in the regulation of Snail. It is difficult to generalise the findings for human cancers, as the pathways described, have been found in different systems (cell culture, animal models, during development, tumour progression, or fibrosis). Given the association between epidermal growth factor receptor (EGFR) over-expression and high metastatic potential, the main questions were:

1) Has EGF an influence on Snail expression in endometrial and ovarian cancer cell lines?
2) If yes, which signalling pathways are involved in the EGF dependent up-regulation of Snail expression?
3) Can the pathways, found in cell culture models, be confirmed in human tumours and metastasis of endometrium and ovary?
4) Are there suitable antibodies to analyse expression of signalling proteins by protein lysate microarrays in lysates of formalin fixed and paraffin embedded tissues?
5) Vary expression profiles of primary tumours and metastases?
6) Which signalling pathways play a role for patient survival?

3 Material and methods

3.1 Reagents and kits

Table 3-1 Reagents, chemicals and Kits used in this thesis

Reagent/Kit	Provider
Acetic Acid	Merck KGaA
40 % Acrylamide/Bis solution 37.5:1	Bio-Rad Laboratories
AG490	Merck KGaA
AG1478	Merck KGaA
Albumin from bovine serum (BSA)	Sigma-Aldrich
Amersham ECL™Advance Western Blotting Detection Kit	GE Healthcare
Amersham ECL Plus Western Blotting Detection Reagents	GE Healthcare
Amersham Hyperfilm™ECL	GE Healthcare
Ammoniumpersulfate (APS)	Sigma-Aldrich
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad Laboratories
Bio-Rad Protein Assay Standard II	Bio-Rad Laboratories
Bromphenol Blue	Sigma-Aldrich
Casein alkalilöslich	Merck KGaA
Chloroform	Merck KGaA
Complete, Mini Protease Inhibitor cocktail tablets	Roche Applied Science
p-Coumaric acid	Sigma-Aldrich
Dako REAL™ Peroxidase Blocking Solution	Dako
Dithiothreitol (DTT)	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™-I	Invitrogen
Dulbecco's PBS without Ca and Mg	PAA
Dimethylsulfoxide (DMSO)	Sigmal-Aldrich
EGF	Merck KGaA
Eosin	Merck KGaA
Ethanol	Merck KGaA
Ethylendiaminetetraacetate (EDTA)	Sigma-Aldrich
FAST®slides	Whatman®Schleicher and Schuell®
Fetal Bovine Serum	PAN Biotech GmbH
Formalin	Merck KGAa
β-Glycerophosphate	Sigma-Aldrich
Glycine	Merck KGaA
Glycerol	Merck KGaA
Hydrogenchloride	Merck KGaA
Hydrogen peroxide (H_2O_2) 30 %	Merck KGaA
2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure (HEPES)	Invitrogen

Reagent/Kit	Provider
Isoamyl alcohol	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Luminol	Alexis Corporation
LY294002	Merck KGaA
Mayer's Hemalaun (Hematoxylin)	Merck KGaA
Magnesiumchloride ($MgCl_2$)	Merck KGaA
Methanol	Merck KGaA
NP-40	Merck KGaA
Paraffin	Merck KGaA
Pertex	Medite
Phosphate buffered saline (PBS)	PAA
Phenylmethylsulfonylfluorid (PMSF)	Sigma-Aldrich
Potassiumchloride	Merck KGaA
POTRAN®-Nitrocellulose Transfer Membrane	Whatman®Schleicher and Schuell®
Precision Plus Dual Colour Protein Standards	Bio-Rad Laboratories
Primer	MWG Biotech
Proteinase K, recombinant, PCR Grade	Roche Applied Science
Probes	Applied Biosystems
Qproteome FFPE Tissue Kit	Qiagen
Random primers	Invitrogen
RNaseOUT™ Recombinant Ribonuclease Inhibitor	Invitrogen
RPMI Medium 1640 GlutaMAX™-I	Invitrogen
SB203580	Merck KGaA
Skim Milk Powder	Sigma-Aldrich
Sodiumchloride (NaCl)	Merck KGaA
Sodium dodecyl sulphate (SDS)	Merck KGaA
Sodiumfluoride	Merck KGaA
Sodiumorthovanadate (Na_3VO_4)	Merck KGaA
Sodiumpyrophosphate ($Na_2P_4O_7$)	Merck KGaA
SuperScript™ II Reverse Transcriptase	Invitrogen
SYPRO®Ruby Protein Blot Stain	Molecular Probes
N, N, N', N'-Tetramethylethan-1,2-diamin (TEMED)	BioRad Laboratories
TaqMan®Universal PCR Master Mix	Applied Biosystems
T-PER™ Tissue Protein Extraction Reagent	Pierce
Tris	Merck KGaA
TRIzol®LS Reagent	Invitrogen
Trypan Blue Stain	Invitrogen
Trypsin-EDTA	PAA
Tween®20	Merck KGaA
UO126	Merck KGaA

3.2 Buffers and solutions

3.2.1 ECL

Solution 1:

100 mM TrisHCl pH 8.6

2.5 mM Luminol

0.4 mM p-Coumaric acid

Solution 2:

100 mM TrisHCl pH 8.6

0.15 % H_2O_2

Mix solutions 1 and 2 short before use

3.2.2 Glycerol storage buffer

50 mM TrisHCl pH 8.3

5 mM $MgCl_2$

0.1 mM EDTA

40 % Glycerol

3.2.3 Hypotonic buffer

10 mM HEPES 7.9

10 mM KCl

0.1 mM EDTA

1.5 mM $MgCl_2$

0.5 mM PMSF

1 mM NaF

1 mM Na_3VO_4

50 mM NaF

10 mM β-Glycerophosphat

10 mM $Na_2P_4O_7$

1 x Complete Mini Protease Inhibitor Mix

3.2.4 Nuclear lysis buffer

10 mM HEPES 7.9

10 mM KCl

0.1 mM EDTA

1.5 mM $MgCl_2$

0.5 mM PMSF

1 mM NaF

1 mM Na_3VO_4

50 mM NaF

10 mM β-Glycerophosphat

10 mM $Na_2P_4O_7$

1 x Complete Mini Protease Inhibitor Mix

3.2.5 Protein lysis buffer

T-PER®Tissue Protein Extraction Reagent

0.5 mM PMSF

1 mM NaF

1 mM Na_3VO_4

50 mM NaF

10 mM β-Glycerophosphat

10 mM $Na_2P_4O_7$

1 x Complete Mini Protease Inhibitor Mix

3.2.6 RNA lysis buffer

10 mM TrisHCl pH 8.0

0.1 mM EDTA

10 % SDS

1 mg/ml Proteinase K

3.2.7 SDS blotting buffer

25 mM Tris

250 mM Glycine

0.1 % SDS

20 % Methanol

3.2.8 10 % SDS resolving gel

For 1 1.5 mm Gel of the Mini-PROTEAN®3 System:

H_2O: 3.7 ml

1 M TrisHCl pH 8.8: 1.9 ml

40 % Acrylamide/Bis solution 37.5:1: 1.9 ml

20 % SDS: 37.5 µl

TEMED: 5 µl

10 % APS: 45 µl

3.2.9 5 % SDS stacking gel

For 1 Gel of the Mini-PROTEAN®3 System:

H_2O: 3.1 ml

1 M TrisHCl pH 6.8: 1.3 ml

40 % Acrylamide/Bis solution 37.5:1: 0.6 ml

20 % SDS: 25 µl

TEMED: 4 µl

10 % APS: 40 µl

3.2.10 5 x SDS gel loading buffer

250 mM TrisHCl pH 6.8

500 mM Dithiothreitol

4 % SDS

0.2 % Bromphenol Blue

20 % Glycerol

3.2.11 SDS gel running buffer

25 mM Tris

250 mM Glycine

0.1 % SDS

3.2.12 Stripping buffer

10 % Methanol

10 % Acetic Acid

3.2.13 TBST

10 mM Tris

150 mM NaCl

0.1 % Tween20

3.3 Equipment

Table 3-2 Equipment used in this thesis

Equipment	Provider
Abi PrismTM 7700 Sequence Detector	Applied Biosystems
Beckman DU®530 Life Science UV/VIS Spektrophotometer	Beckman Coulter GmbH Diagnostics
BioOdyssey™Calligrapher™MiniArrayer	Bio-Rad Laboratories
BRANSON Digital Sonifier®	Branson
Eppendorf centrifuge 5417R	Eppendorf
EAGLE EYE™II	Stratagene
HPScanjet3770	Hewlett-Packard Development Company
Mini-PROTEAN®3	Bio-Rad Laboratories
MicroCaster™ Slide Microarrayer	Schleicher&Schuell
Mikrotome HM335E	Microtech
Lichtmikroskop LABOVERT FS	Leitz
PCR Cycler Primus 96 Plus	MWG Biotech
Powersupply	Biometra
Sigma centrifuge 4K15	Sartorius AG
Tabletop processor SRX-101A	Konica-Minolta
Thermomixer comfort 5355 R	Eppendorf
Tissue Block System TBS88 Dispenser Unit	Medite Medizintechnik
Waterbath GFL®	Hilab

3.4 Software

Table 3-3 Software used in this thesis

Software	Provider
Cluster (version 2.11)	www.rana.lbl.gov
Tree View (version 1.6)	www.rana.lbl.gov
Microsoft Excel (version 2002)	Microsoft
ScionImage (version 0.4.0.3.)	ScionCorporation
SPSS for Windows (version 16.0)	SPSS Inc.

3.5 List of providers

Applied Biosystems, Darmstadt, Germany

Alexis Corporation, Lausen, Switzerland

Beckman Coulter GmbH, Krefeld, Germany

Biometra, Goettingen, Germany

Bio-Rad Laboratories, Muenchen, Germany

Branson, Chicago, USA

DakoCytomation, Glostrup, Denmark

Eppendorf AG, Hamburg, Germany

GE Healthcare, Buckinghamshire, UK

Hewlett-Packard, Dornach, Germany

Hilab, Duesseldorf, Germany

Invitrogen, Karlsruhe, Germany

Konica-Minolta, Langenhagen, Germany

Leitz, Bensheim, Germany

Medite Medizintechnik, Burgdorf, Germany

Merck KGaA, Darmstadt, Germany

Microsoft, Unterschleissheim, Germany

Microtech, Franchville, France

New England Biolabs, Frankfurt am Main, Germany

MWG Biotech, Ebersberg, Germany

PAA Laboratories, Pasching, Austria

Qiagen, Hilden, Germany

Roche Applied Science, Mannheim, Germany

Sartorius AG, Goettingen, Germany

Scion Corporation, Maryland, USA

SPSS Inc., Chicago, USA

Stratagene, La Jolla, USA

Whatman®Schleicher and Schuell®, Dassel, Germany

3.6 Cell Culture

3.6.1 Cell Lines and Growth Media

Table 3-4 Growth conditions for cell lines used

Name	Tissue	Growth medium	Property, morphology
Ishikawa (ECACC No. 99040201)	human endometrial adenocarcinoma	DMEM 10 % FBS	adherent epithelial
Ishikawa ER- (ECACC No. 98032302)	human endometrial adenocarcinoma	DMEM 10 % FBS	adherent epithelial
MCF-7 (ECACC No. 86012803)	human breast carcinoma	DMEM 10 % FBS	adherent epithelial
OVCAR-3 (ATCC® No. HTB-161™)	human ovarian adenocarcinoma	RPMI 1640 20 % FBS 0.01 mg/ml insulin	adherent epithelial

3.6.2 Cell culture conditions

Cells were grown at 37 ºC in an atmosphere of 5 % CO_2.

3.6.3 Preparation of frozen stocks

Cell lines were trypsinized, washed in medium and centrifuged for 5 min at 500 x g. The pellet was resuspended in FBS supplemented with 10 % DMSO. The cells were stored at -20 ºC for 6 hours, at – 80 ºC for 24 hours and were then transferred in liquid nitrogen for long-time storage.

3.6.4 Starting cultures from frozen stocks

Vials with frozen cells were transferred to a 37 ºC water bath. After thawing, the cells were transferred to the medium containing tubes, washed twice with medium and then seeded in cell culture flasks.

3.6.5 Passaging of cells

Cells were washed with PBS, trypsinized until cells detached from the plastic, harvested in medium, diluted in an appropriate ratio and seeded in cell culture flasks.

3.7 Protein kinase inhibitors and EGF stimulation

The protein kinase inhibitors AG1478, SB203580, UO126 and AG490 were used at a concentration of 10 µM, LY294002 was used at a concentration of 20 µM. Cells were serum starved for 24 hours and incubated for 60 minutes prior to stimulation with EGF used at a concentration of 100 ng/ml.

3.8 Tissues

3.8.1 Endometrial cancer

Seventeen formalin fixed and paraffin embedded primary endometrial adenocarcinomas (endometrioid subtype, WHO) diagnosed between 1992 and 2003 were selected from the archive of the Institut fuer allgemeine Pathologie und Pathologische Anatomie, Technische Universitaet Muenchen, Germany. Some cases were obtained during intrauterine curetting. Grading and staging of the primary carcinomas were ascertained according to the proposals of the WHO and the UICC. The study was approved by the Ethics Committee of the Technische Universitaet Muenchen. Clinicopathological parameters of the patients are summarized in **Table 3-5**.

Table 3-5 Clinicopathological characteristics of endometrial cancer patients

No.	Tumour grade	UICC	Age (years)
1	2	1c	77
2	3	Tx	76
3	2	Tx	89
4	3	3a	66
5	3	1a	75
6	2	2b	56
7	2	3a	85
8	2	1c	69
9	2	2b	58
10	1	3a	77
11	1	3c	70
12	1	1b	88
13	2	2b	65
14	3	3a	63
15	3	2a	67
16	3	1c	37
17	2	1b	70

3.8.2 Ovarian cancer

Formalin-fixed and paraffin embedded (FFPE) tissue samples from a total of 25 patients who had undergone primary surgery for newly diagnosed advanced stage (FIGO IIIC and IV) ovarian carcinoma were included in this study. The clinicopathological characteristics of the patients are summarized in **Table 3-6**. The study was approved by the Ethics Committee of the Technical University of Munich, Germany. Only patients without chemotherapy or radiotherapy within 6 month prior to surgery were included in the study. Follow-up data were available for 24 patients, with a median follow-up time of 55 months. The median follow-up was calculated by the inverse Kaplan-Meier approach (Schemper & Smith, 1996). During follow-up time 16 of 24 patients died. From one patient (no. 18) no follow-up data were available. Therefore, this patient was excluded for the correlation with the clinical parameters and survival but included in the cluster analysis. From the same patients, metastases that were located in the peritoneum,

omentum, distant lymph nodes or uterus, were analysed. From one metastasis (from patient 25) not enough protein could be extracted.

Table 3-6 Clinicopathological characteristics of ovarian cancer patients

No.	Subtype	Tumour grade	T	N	M	Figo	status	Survival / last follow up (months)	age
1	serous	3	3c	1	0	3c	dead	19.7	52.8
2	serous	3	3c	x	1	4	dead	3.3	80.0
3	serous	3	3b	1	0	3c	alive	30.6	74.8
4	serous	2	3c	x	0	3c	dead	26.5	52.1
5	serous	2	3c	0	0	3c	dead	23.4	69.5
6	endometrioid	3	3c	1	0	3c	dead	32.4	47.1
7	serous	3	3c	x	0	3c	alive	0.1	80.2
8	serous	3	3c	x	1	4	alive	54.7	55.4
9	serous	3	3c	1	1	4	dead	0.8	49.3
10	serous	3	3c	x	0	3c	dead	17.9	80.2
11	endometrioid	3	3c	x	1	4	dead	16.2	55.4
12	serous	3	3c	1	0	3c	alive	56.8	73.6
13	serous	2	3c	1	0	3c	dead	55.7	29.5
14	serous	3	3c	1	0	3c	alive	47.7	64.2
15	serous	3	3c	1	0	3c	dead	14.0	65.3
16	serous	3	3c	1	x	3c	alive	30.7	68.8
17	other	3	3c	1	0	3c	dead	5.5	58.9
18	serous	1	3c	x	x	3c	n.d.	n.d.	71.4
19	serous	3	3c	x	1	4	dead	6.4	71.6
20	serous	2	3c	0	0	3c	dead	50.0	36.9
21	serous	3	3c	1	0	3c	dead	31.2	41.2
22	other	3	3c	x	0	3c	alive	49.2	61.1
23	serous	3	3c	x	0	3c	dead	17.7	78.2
24	serous	3	3c	1	0	3c	dead	48.8	48.3
25	serous	1	3c	x	0	3c	alive	55.0	76.8

n.d. no data available

3.8.3 H&E staining

After standard deparaffination (xylene, isopropanol, ethanol 96 %, ethanol 70 %, 20 minutes each) sections were stained for five minutes in Haematoxylin, subsequent five minutes in tap water and then five minutes in Eosin. Past dehydration of the sections they were covered with Pertex.

3.9 Protein extraction

3.9.1 Protein extraction from cell lines

The monolayer was washed with PBS, cells were lysed with protein lysis buffer (100 µl per 6 well) and transferred to a 1.5 ml tube. Lysates were incubated for 20 minutes on ice and centrifuged for 20 minutes at 20000 x g at 4 ºC. The supernatant was transferred to a fresh tube and stored at -20 ºC.

3.9.2 Cell fractionation

Cell pellets were lysed in hypotonic buffer (300 µl per T-75 flask) and incubated for 3 minutes on ice. Afterwards NP-40 up to an end concentration of 0.1 % was added and cells were vortexed until complete lysis. Cell lysis was controlled by Trypanblue staining. Then the lysate was centrifuged for 4 minutes and 400 x g at 4 ºC, cytoplasmatic supernatant was transferred to a new tube and centrifuged at 20000 x g for 10 minutes. The nuclei were washed three times in glycerol storage buffer each followed by a centrifugation step for 4 minutes at 400 x g. After incubation for 30 minutes in nuclear lysis buffer, nuclei were sonificated for 45 seconds at 70 % output, again incubated for 15 minutes on ice and finally centrifuged at 20000 x g for 10 minutes and supernatant was transferred into a new tube.

3.9.3 Protein extraction from formalin fixed and paraffin embedded tissues

For protein extraction from formalin fixed and paraffin embedded tissues the Qproteome FFPE Tissue Kit was used. Tumour areas containing at least 85 % tumour cells were selected and marked on an H&E stained reference slide. After standard deparaffination of the 10 µm tissue sections, tumour areas were scratched from the unstained slides and transferred into Qproteome Extraction Buffer. The samples were incubated 20 minutes at 100 ºC and 2 hours at 80 ºC with agitation at 750 rpm. After incubation samples were centrifuged at 20000 x g, supernatant was transferred into a new safe-lock tube and stored at -20 ºC.

3.10 Protein quantification

3.10.1 Bradford assay

Bio-Rad standard II was diluted to concentrations between 1.6 and 50 µg/ml, samples were diluted 1:100, Bio-Rad Protein Assay Dye Reagent Concentrate was diluted 1:5 with distilled water. For the assay 100 µl of prepared standards or samples were mixed with 900 µl of diluted Dye and optical density was measured at 595 nm.

3.10.2 SYPRO®Ruby protein blot stain

Protein lysates were spotted on nitrocellulose coated glass slides as described in **chapter 3.13.1**. All following incubation steps were performed with gentle agitation on an orbital shaker at 30 rpm. Slides were washed in TBST-Buffer for 5 minutes. The slides were floated in 7 % acetic acid, 10 % methanol for 15 minutes, washed in four changes of deionised water for 5 minutes each, stained with SYPRO®Ruby Protein Blot Stain reagent for 15 minutes and washed again in 4 changes of deionised water for 1 minute each. The wet slides were immediately monitored and photographed on an Eagle-Eye®II UV transilluminator.

3.11 Antibodies

Table 3-7 Primary antibodies used for Immunoblotting and protein lysate microarrays

Protein	Size MW (kDa)	Clone	Company	Blocking buffer	Dilution
Phospho-AKT (Ser473)	60	#9271	NEB	5 % MP	1:1000*
AKT (pan)	60	C67E7, #4691	NEB	5 % MP	1:1000*
β-Actin	42	AC-15	Sigma	5% MP	1:5000
Cytokeratin 18	46	DC10, #4548	NEB	5 % MP	1:1000*
E-cadherin	120	36 (AEC)	BD	5 % MP	1:5000
Phospho-ERα (Ser118)	66	(16J4), #2511	NEB	5 % MP	1:1000*
ERα	67	578-595	Sigma	0.5% Casein	1:3000
Phospho-ERK1/2 (Thr202/Tyr204)	42, 44	#9101	CST/NEB	5% MP	1:1000*
ERK1/2	42, 44	#9102	NEB	5 % MP	1:1000*
Phospho-EGFR (Tyr1086)	170-180	ZMD.504	Invitrogen	5 % MP	1:5000*
EGFR	170	#2232	NEB	5 % MP	1:2000*
Phospho-GSK-3β (Ser9)	46	#9336	NEB	5 % MP	1:1000*
GSK-3β	46	27C10, #9315	NEB	5 % MP	1:1000*
Phospho-HER2 (Tyr1248)	185	#44-900	Invitrogen	5%MP	1:1000*
HER2	185	Code A0485	Dako	5 % MP	1:1000*
Histone H3	17	#9715	NEB	5 % MP	1:5000
Phospho-HSP27 (Ser82)	27	#2401	NEB	5 % MP	1:1000*
HSP27	27	G31, #2402	NEB	5 % MP	1:1000
Phospho-NF-κB p65 (Ser536)	75	#3031	NEB	5 % MP	1:1000*
NF-κB p65	75	#3034	NEB	5 % MP	1:1000*
Phospho-p38 MAPK (Thr180/Tyr182)	43	12F8, #4631	NEB	5 % MP	1:1000*
p38 MAPK	43	#9212	NEB	5 % MP	1:1000*
Phospho-PAK1(Thr 212)	68	PK-18	Sigma	5 % MP	1:1000*
PAK1	68	#2602	CST	5 % MP	1:1000*
PTEN	54	#9552	NEB	5 % MP	1:1000*
Snail	29	9H2-1-1	Dr. Kremmer (Rosivatz et al., 2006)	2.5 % MP + 0.5 % BSA	1:2500
Phospho-STAT3 (Tyr705)	79, 86	D3A7, #9145	NEB	5 % MP	1:1000*
STAT3	79, 86	(79D7)	NEB	5 % MP	1:1000*
α-Tubulin	50	DM 1A	Sigma	5 % MP	1:5000
Twist1	26	#4119	NEB	5 % MP	1:1000*
Vimentin	57	V9	Dako	5 % MP	1:1000

* antibodies were diluted in 5 % BSA; remaining antibodies were diluted in blocking buffer;

Table 3-8 HRP conjugated secondary antibodies

Antibody	Company	Dilution
ECL Mouse IgG, HRP-Linked Whole Ab	GE Healthcare	1:5000
ECL Rat IgG, HRP-Linked Whole Ab	GE Healthcare	1:5000
Anti-rabbit IgG, HRP-Linked Ab	New England Biolabs	1:2000

3.12 Immunoblotting

3.12.1 SDS gel electrophoresis

Protein lysates were supplemented with SDS Gel Loading Buffer and 50 to 100 µg of extracts were subjected to SDS polyacrylamide gel electrophoresis. Proteins were separated for 90 minutes at 100 V.

3.12.2 Blotting

Proteins were transferred for 90 minutes at 100 V to a PROTRAN®Nitrocellulose Transfer Membrane.

3.12.3 Detection

Membranes were first blocked for one hour at room temperature to avoid unspecific antibody binding (blocking buffer see **Table 3-7**). Primary antibodies (**Table 3-7**) were incubated for 16 hours at 4 ºC. After washing three times with TBST, membranes were incubated for 30 minutes with appropriate horseradish peroxidase (HRP) linked secondary antibodies (**Table 3-8**), diluted in blocking buffer. After washing three times in TBST, Immunoblots were developed with ECL, ECLPlus™ Western Blotting Detection Reagent and ECL™Advance Western Blotting Detection Kit, respectively, on an Amersham Hyperfilm™ECL.

3.12.4 Stripping

Membranes were washed twenty minutes in stripping buffer and 5 minutes in TBST.

3.13 Protein lysate microarrays

Western blot analysis cannot easily be performed in a high throughput manner and requires a large amount of test material, what is not possible to obtain from clinical tissue samples or biopsies. Therefore I used protein lysate microarrays for profiling protein networks in endometrial and ovarian cancer.

After protein extraction from formalin fixed and paraffin embedded tissues, each patient lysate was immobilized on nitrocellulose coated glass slides in a miniature dilution curve, so that each analyte/antibody combination could be analysed in the linear dynamic range of the system. Specific proteins were detected by specific antibodies and visualized by chemiluminescence, protein expression was quantified and analysed by statistical and cluster analysis (**Figure 3.1, Figure 3.2**).

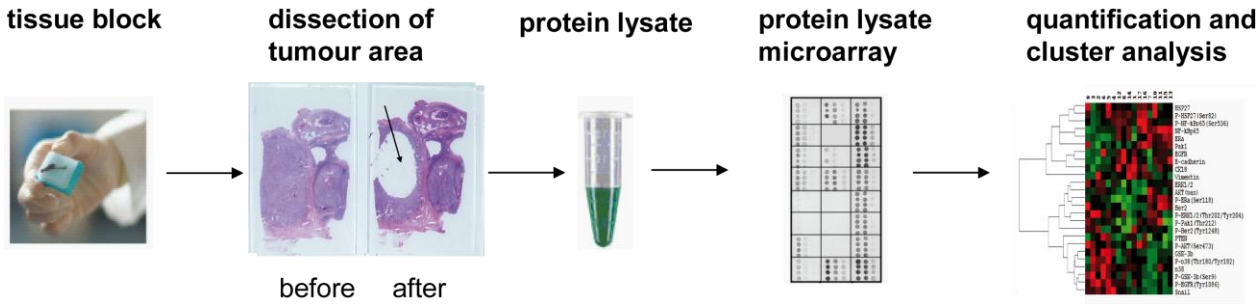

Figure 3.1 Flow chart of protein lysate microarrays from formalin fixed and paraffin embedded tissues. 10 µm sections were cut from paraffin embedded tissues. Tumour areas were dissected with a needle and protein lysates were prepared. Potein lysates were applied onto nitrocellulose coated glass slides (FAST^{TM} Slides). Specific proteins were detected using specific antibodies and slides were developed using chemiluminescence. Finally protein expression was quantified and statistical analysis and cluster analysis was performed.

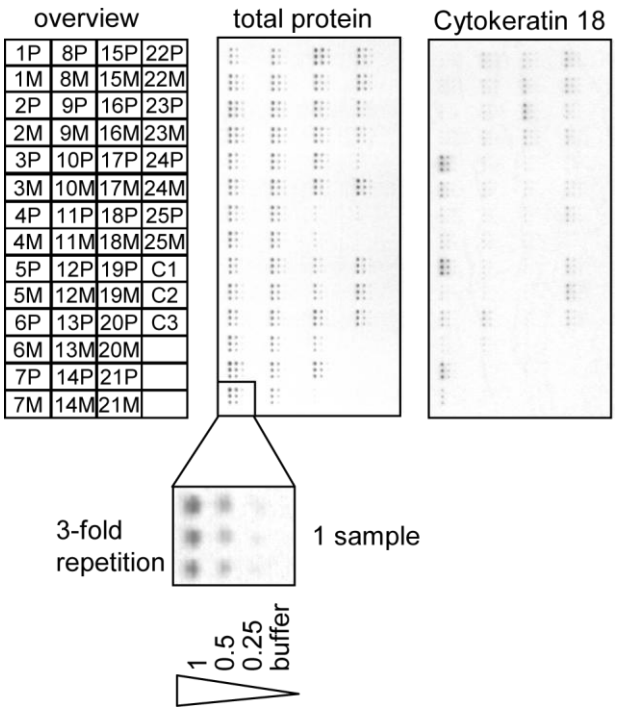

Figure 3.2 Example for protein lysate microarrays stained for Sypro Ruby (total protein) and Cytokeratin 18. 25 primary tumours (P) and corresponding metastases (M) of 25 ovarian cancer patients were analysed. From every sample the undiluted lysate, two dilutions (1:2, 1:4) and a negative control (extraction buffer only) were applied in three replicates onto FAST™Slides. Total protein was determined by Sypro Ruby® Protein Blot Stain. Proteins were detected using specific antibodies and slides were visualized using chemiluminescence. Protein expression was normalised to total protein and statistical and cluster analysis was performed. As controls, pools of lysates from primary tumours (C1), metastases (C2) and primary tumours and metastases (C3) were analysed.

3.13.1 Spotting

Protein lysates (1mg/ml) were spotted onto FAST® Slides using a MicroCaster™ Slide Microarrayer (for the endometrial cancer study) and a BioOdyssey™Calligrapher™-MiniArrayer (for the ovarian cancer study). For every lysate a 3-fold repetition and a dilution curve (undiluted, 1:2, 1:4 and buffer) were spotted.

3.13.2 Detection

Slides were first blocked in Dako REAL™Peroxidase Blocking Solution. Protein blocking and Antibodies see **chapter 3.11**. Exposure time of films was chosen, so that the undiluted lysate of each sample was still in the linear range.

3.13.3 Quantification

For estimation of total protein amounts, parallel arrays were stained with Sypro Ruby®Protein Blot Stain as described in **chapter 3.10.2**. Visualised arrays were scanned on a scanner with 1200 dpi and saved as tiff files. Densities of protein spots (of the undiluted lysate) were measured using Scion Image.

3.14 RNA extraction

3.14.1 RNA extraction from cell lines

Cell monolayers in six well plates were washed in cold PBS scraped off in 0.5 ml TRIzol®LS Reagent and incubated for five minutes at room temperature. RNA was purified by chloroform extractions. Therefore 100 µl of chloroform were added, vortexed, incubated for ten minutes and centrifuged for five minutes at 18000 x g. RNA was precipitated with an equal volume of isopropanol. The RNA pellet was washed twice in 70 % ethanol, air dried and resuspended in 25 µl water. RNA was stored at -70 ºC. For quantification of RNA concentration OD260/280 was determined.

3.14.2 RNA extraction from formalin fixed and paraffin embedded tissues

After standard deparaffination of 8 µm tissue sections, tumour areas containing at least 85 % of tumour cells were dissected with a needle (as described in **chapter 3.9.3**), transferred to RNA-Lysis-Buffer and incubated for 16 hours at 60 ºC. RNA was purified by phenol chloroform extractions (one volume of phenol pH 4.5, chloroform, isoamylalcohol in a proportion of 25:24:1) followed by precipitation with an equal volume of isopropanol as described above in chapter **3.14.1**.

3.15 cDNA synthesis

1 µg RNA, 1 µl of Random Primers and 1 µl of dNTPs (10mM each) were incubated 2 minutes at 70 ºC and 10 min at 25 ºC. Following 4 µl First Strand Buffer, 2 µl DTT (0.1 M), 1 µl SuperScript™ II Reverse Transcriptase (200 Units per µl) and 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor (40 Units per µl) were added to the reaction. First strand synthesis was performed for 60 minutes at 42 ºC and 15 minutes at 70 ºC. cDNA was stored at -20 ºC.

3.16 Real Time Quantitative RT-PCR

3.16.1 Primers and Probes for real time quantitative RT-PCR

Table 3-9 Primers and Probes used for Real time quantitative PCR (Rosivatz et al., 2002)

Gene	Forward Primer	Reverse Primer	Probe
GAPDH	cgtggaaggactcatgacca	gccatcacgccacagtttc	cagaagactgtggatggcccctcc
E-cadherin	gaacagcacgtacacagccct	gcagaagtgtccctgttccag	atcatagctacagacaatggttctccagttgct
Snail	tgcaggactctaatccaagtttacc	gtgggatggctgccagc	tccagcagccctacaccaggcc

3.16.2 Real Time Quantitative PCR

Real-time quantitative RT-PCR analysis was performed using ABI PRISM 7700 Sequence Detection System. Primers and Probes are shown in **Table 3-9**. Probes were modified with the reporter dye 6-FAM on the 5' end and quencher dye TAMRA at the 3' end. Real time RT-PCR was performed with the TaqMan®Universal PCR Master Mix using 2 µl of cDNA, 150 µM of probe and primers in a 20 µl final reaction mixture. After a two minute incubation at 50 ºC and ten minutes at 95 ºC, 40 PCR cycles, each consistent of 15 seconds at 95 ºC and one minute at 60 ºC were conducted.

3.16.3 Quantification of Expression

Relative expression levels of target sequences were determined by the standard curve method using a standard cDNA solution of MCF-7 cells. Expression of Snail and E-cadherin was normalised to expression levels of the housekeeping gene GAPDH.

3.17 Statistical Analysis

3.17.1 Spearman rank correlation test

Bivariate relationships were analysed by the Spearman rank correlation test, using SPSS version 16.0 (SPSS, Inc, Chicago, IL). To reduce multiple test issue Bonferroni adjustment was conducted in the multiple correlation analyses regarding Snail to warrant a global significance level of 5%.

3.17.2 Cox-Proportional-Hazard-Models

Statistical analyses were carried out with the software package R Version 2.7.1.
As the main interest was on examining the influence of risk factors on survival, Cox-Proportional-Hazard-Models were fit to the data. Hazard Ratios (HR) describing the factorial change of risk by a one-point increment on the scale of a risk factor were calculated and reported with 95% confidence intervals (CI). As the biological pathway suggested relations between several factors regarding survival, interaction terms were included in the model process. The introduction of interactions allows the observer to examine mutual effects of risk factors. To illustrate the examined influence on risk and therefore facilitate the interpretation of the model parameters, surface-plots for the Hazard Ratio were created. Statistical survival analyses were conducted at a 0.05 level of significance.

3.18 Cluster Analysis

Unsupervised hierarchical clustering (Average Linkage Clustering, Spearman Rank Correlation) was performed by using Cluster and Tree View software (Eisen et al., 1998). Proteins were weighted equally.

4 Results

4.1 Regulation of the E-cadherin repressor Snail in endometrial carcinoma

4.1.1 EGF regulates Snail expression in Ishikawa and Ishikawa ER- cells

To address the question whether EGFR activation effects Snail protein and/or mRNA expression, the human endometrial carcinoma cell lines Ishikawa and Ishikawa ER- were examined. Both cell lines have been established from an endometrial adenocarcinoma; the Ishikawa cells derive from the uterus, while the origin of the Ishikawa ER- cell line is unknown. Both cell lines show an epithelial morphology and are EGFR positive but it is not known, if the cell lines descend from the same patient.
EGF stimulation of Ishikawa and Ishikawa ER- cells led to up-regulation of Snail protein expression. Snail was localised in the nucleus only, as revealed by fractionated protein extraction (**Figure 4.1**).

To examine if EGFR signalling also affects Snail mRNA expression levels, quantitative real time RT-PCR analysis of EGF stimulated cells was performed. Surprisingly Snail mRNA levels were only affected in Ishikawa cells; in Ishikawa ER – cells EGF stimulation had no influence on Snail mRNA levels. In both cell lines E-cadherin expression levels were not influenced by up-regulation of Snail (**Figure 4.2**).

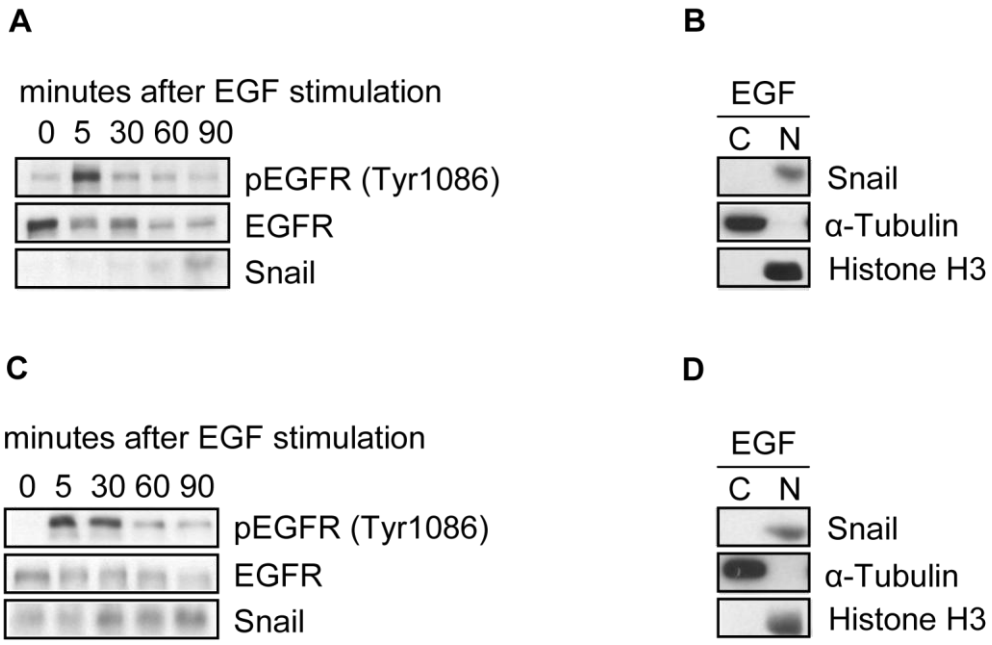

Figure 4.1 Western blot analysis of Ishikawa (A, B) and Ishikawa ER- (C, D) cells. (A, C) Cells were stimulated with EGF for 5, 30, 60 and 90 minutes before protein extraction and 1D gel electrophoresis. **(B, D)** Cells were stimulated with EGF for 90 minutes before cell fractionation into cytoplasmatic (C) and nuclear (N) fractions. Proteins analysed are indicated on the right. One of three independent experiments is shown.

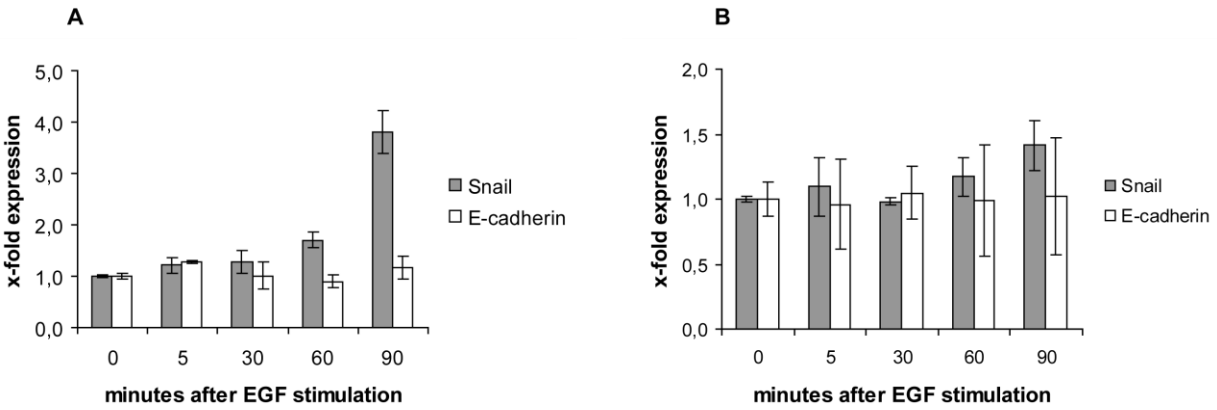

Figure 4.2 Snail and E-cadherin mRNA expression of Ishikawa (A) and Ishikawa ER- (B) cells. Cells were stimulated with EGF for 5, 30, 60 and 90 minutes before RNA extraction. Data were normalised to time point zero (without stimulation). The mean and standard deviation of three independent experiments, each of which was performed in duplicates, are shown.

To identify the pathways leading to Snail protein up-regulation after EGF stimulation EGFR, p38 MAPK, MEK/ERK and PI3K pathways were inhibited (**Figure 4.3**).

Therefore serum starved cells were pre-treated for 60 minutes with Inhibitors to EGFR (AG1478), p38 MAPK (SB203580), MEK/ERK (UO126), and PI3K (LY294002) and subsequent stimulated with EGF for 90 minutes.

In the Ishikawa cells (**Figure 4.3 A**) the ERK pathway was found to be involved in the EGF dependent up-regulation of Snail protein expression. Inhibition of EGFR prevented also phosphorylation of AKT, NF-κBp65 and PAK1. But inhibition of PI3K did not influence Snail expression. Inactivation of GSK-3β and activation of HSP27 was not influenced by EGF.

In the Ishikawa ER- cells (**Figure 4.3 B**), both AKT and ERK pathways contributed to the up-regulation of Snail protein expression after EGF stimulation. Inhibition of EGFR prevented also phosphorylation of p38 MAPK, HSP27 and NF-κBp65. But inhibition of p38 MAPK did not lead to reduction of Snail expression. Inactivation of GSK-3β was not influenced by EGF.

Inhibition of EGFR and MEK/ERK pathways in Ishikawa cells led – as already seen for the protein expression – also to a reduction of Snail mRNA (**Figure 4.4**).

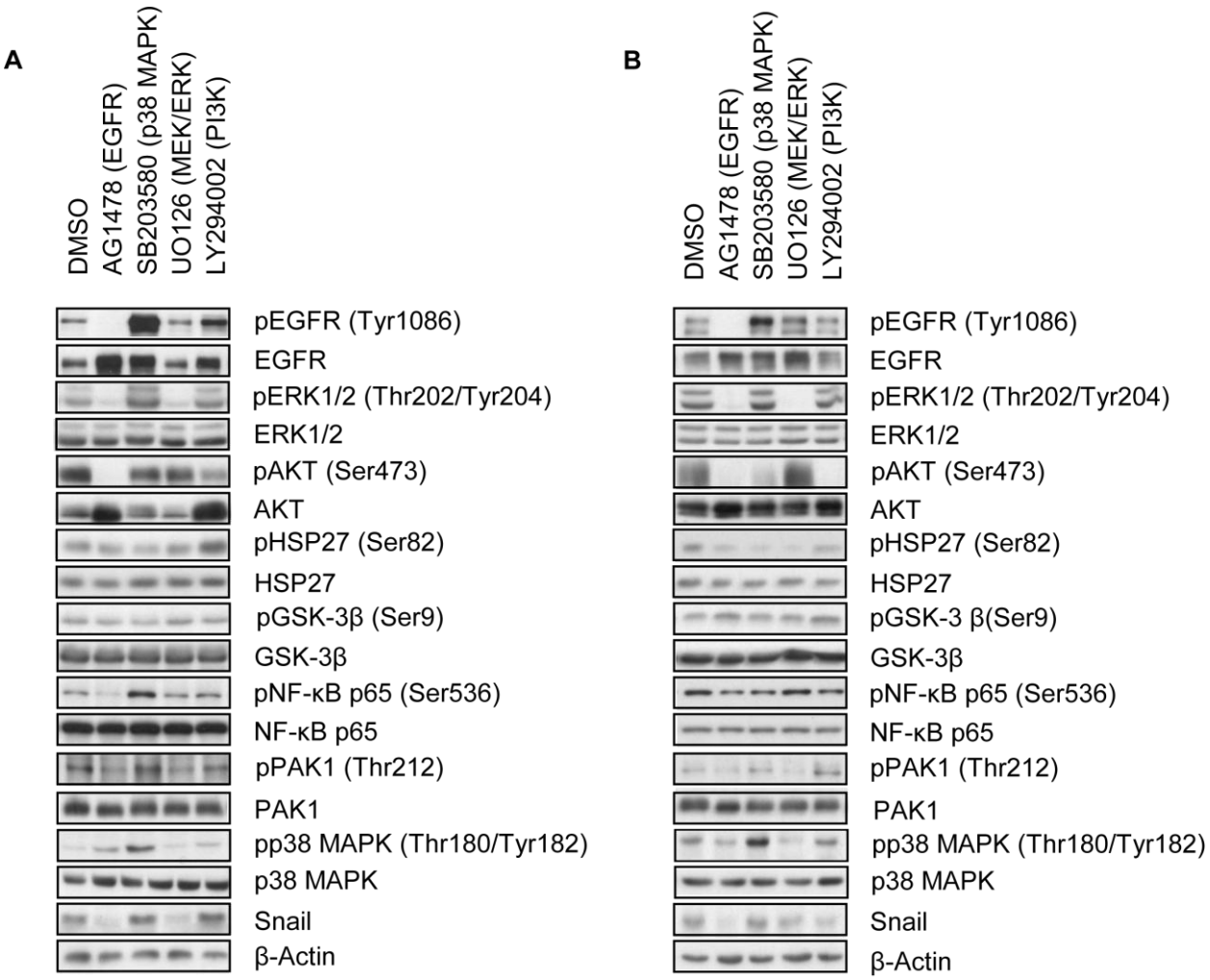

Figure 4.3 Western blot analysis of Ishikawa (A) and Ishikawa ER- (B) cells. Cells were incubated with the specific protein kinase inhibitors AG1478 (inhibits EGFR), SB203580 (inhibits p38 MAPK), UO 126 (inhibits MEK/ERK) and LY294002 (inhibits PI3K) each dissolved in DMSO, respectively, 60 minutes prior to stimulation with EGF for 90 minutes. One of three independent experiments is shown.

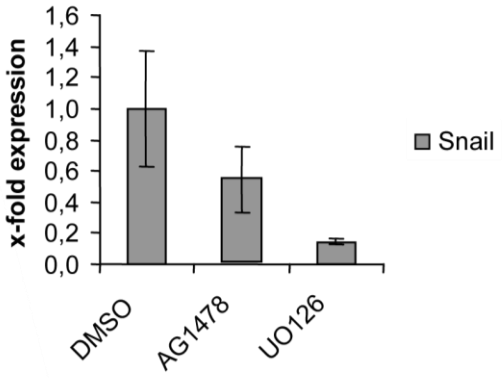

Figure 4.4 Snail mRNA expression in Ishikawa cells after inhibiting the EGFR and ERK1/2 pathways. Cells were incubated 60 minutes with AG1478 (inhibits EGFR) and UO126 (inhibits MEK/ERK), respectively, before stimulation with EGF for 90 minutes. The mean and standard deviation of three independent experiments, each of which was performed in duplicates, are shown.

4.1.2 Profiling of signalling pathways in endometrial primary tumours using protein lysate microarrays

4.1.2.1 Validation of antibodies for lysate microarrays

The specificities of antibodies used for protein lysate microarrays have to be tested by western blot analysis. All 27 antibodies used in this thesis were tested with lysates from endometrial, breast or ovarian carcinoma (**Figure 4.5**). Testing revealed that antibodies suitable for protein microarray analysis can be divided into three groups. 18 of 27 (67 %) antibodies (**Figure 4.5 A**) showed either one specific band with expected molecular weight (pAKT, AKT, E-cadherin, Cytokeratin 18, pERα, pGSK-3β, GSK-3β, pHER2, HSP27, NF-κp65, pSTAT3, pPAK1, PAK1, PTEN and p38 MAPK), or two bands with known molecular weights (HER2, pERK1/2, ERK1/2). 7 of 27 (26 %) antibodies (**Figure 4.5 B**) showed more than one band in the western blot but intensities of all bands correlated with the intensity of the specific band in the western blot (pEGFR, EGFR, pHSP27, Snail, ERα, pNF-κBp65 and pp38 MAPK). Only 2 of 27 (7 %) antibodies (**Figure 4.5 C**) showed additional bands in the western blot, which did not correlate with the band at the expected molecular weight (Vimentin, STAT3). Nevertheless, the signal of the protein lysate microarray correlated with the signal of the specific band in western blot analysis, suggesting that the cross-reactive band may be associated with 1D gel electrophoresis; therefore these antibodies were included into the study as well.

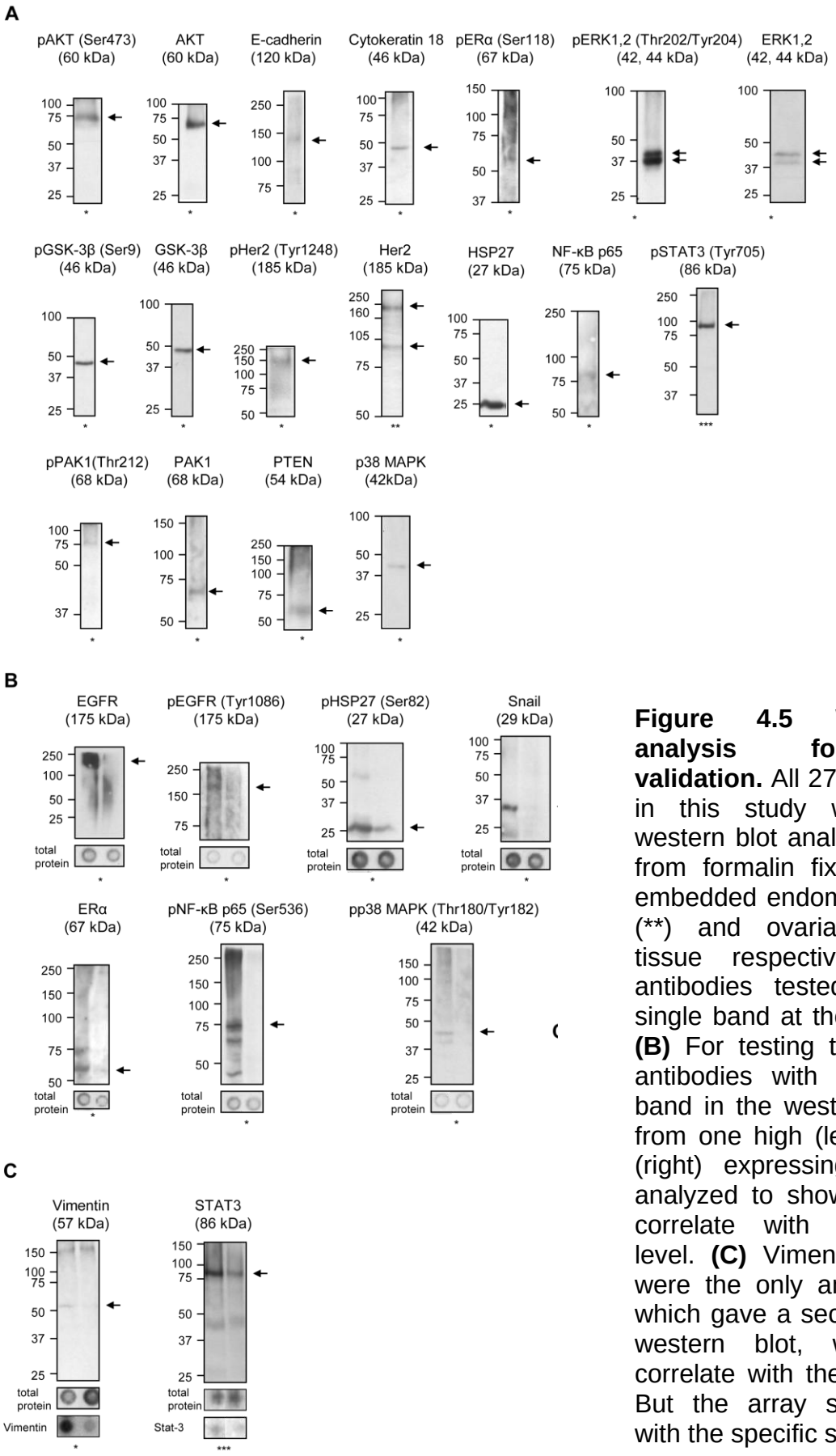

Figure 4.5 Western blot analysis for antibody validation. All 27 antibodies used in this study were tested in western blot analysis with lysates from formalin fixed and paraffin embedded endometrial (*), breast (**) and ovarian (***) cancer tissue respectively. **(A)** Most antibodies tested, showed one single band at the expected size. **(B)** For testing the specificity of antibodies with more than one band in the western blot, lysates from one high (left) and one low (right) expressing tumour were analyzed to show that all bands correlate with the expression level. **(C)** Vimentin and STAT-3 were the only antibodies tested, which gave a second band in the western blot, which did not correlate with the specific signal. But the array signal correlated with the specific signal.

4.1.2.2 Molecular profiling of primary endometrial tumours

To answer the question whether EGFR activation may be involved in Snail regulation in primary endometrial cancers, protein lysate microarray technology was applied. After having demonstrated the specificities of all used antibodies (**chapter 4.1.2.1**), protein lysate microarray analysis with protein extracts from seventeen FFPE endometrial cancers was applied, in order to correlate Snail protein expression to abundances of a number of signalling proteins.

Unsupervised hierarchical cluster analysis revealed that the samples were divided into two groups (**Figure 4.6**). The first group shows Snail clustering close to the phosphorylated forms of EGFR and GSK-3β; the phosphorylated forms of p38 MAPK, AKT, HER2, PAK1, ERK1/2, ERα, the non-phosphorylated forms of PTEN, GSK-3β, p38 MAPK, HER2, AKT, ERK1/2 and Snail mRNA are also part of this cluster. The second cluster is composed of E-cadherin, Cytokeratin 18, the non-phosphorylated forms of EGFR, PAK1 ERα, NF-κBp65, HSP27 and the phosphorylated forms of NF-κBp65, HSP27.

In primary endometrial tumours a relation between Snail protein expression and the phosphorylated form (Tyr1086) of EGFR was seen. In addition, the active, phosphorylated forms of p38 MAPK, AKT, HER2, PAK1 and ERK1/2 and the inactive, phosphorylated form of GSK-3β may be involved in the regulation of Snail after activation of EGFR. We also see an inverse correlation between Snail and the two epithelial markers E-cadherin and Cytokeratin 18. The non-phosphorylated form of ERα showed an inverse correlation with Snail protein expression. NF-κBp65 and HSP27 also show an inverse correlation with Snail and seem not to be involved in EGFR dependent regulation of Snail.

Due to insufficient sample size, no correlation with clinicopathological parameters (tumour grade, UICC and age) was done, because I was not able to conduct group comparisons.

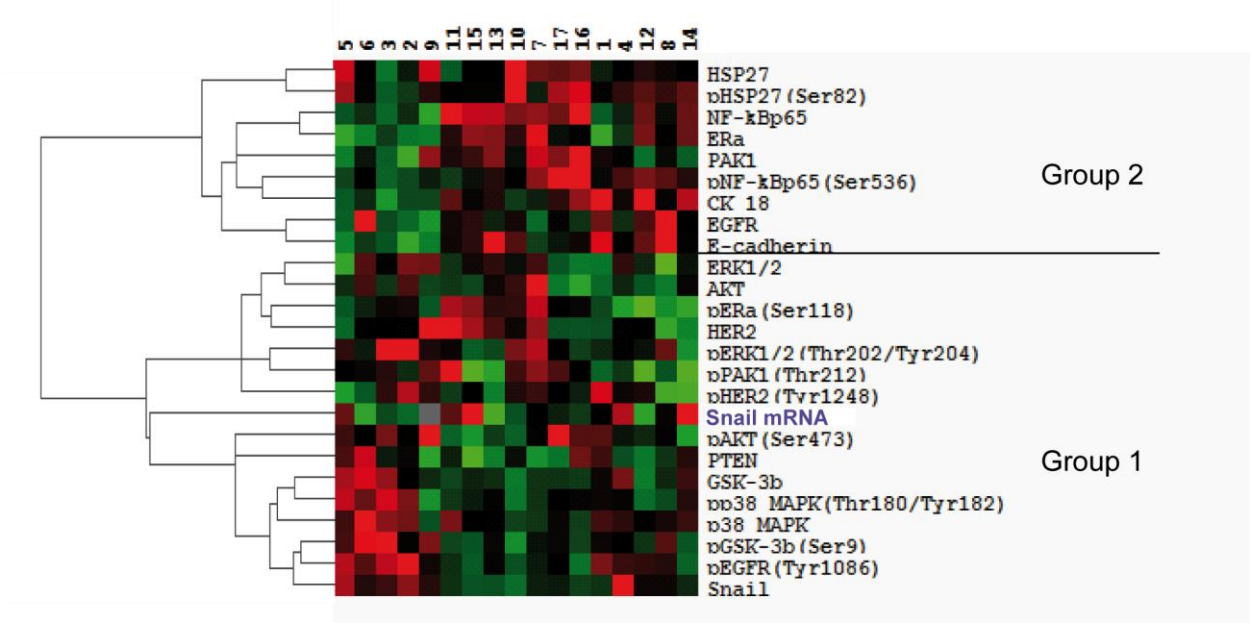

Figure 4.6 Unsupervised hierarchical clustering image map. Twenty-four proteins (indicated in black) and Snail mRNA (indicated in blue) were used in the analysis of 17 endometrial primary tumours. Two different molecular network groups were identified and are indicated on the right. The patients are numbered in the horizontal axis and the protein expression is indicated on the vertical axis. Higher protein expression levels are represented in red, lower levels in green. For patient No. 9 RNA extraction was not successful (indicated in grey).

4.2 Regulation of the E-cadherin repressor Snail in ovarian carcinoma

4.2.1 EGF regulates Snail expression in OVCAR-3 cells

To address the question whether EGFR activation effects Snail protein and/or mRNA expression, in a second cancer type, the human ovarian carcinoma cell line OVCAR-3, which has been established from the malignant ascites of a patient with progressive adenocarcinoma of the ovary, has been examined. EGF stimulation of serum starved OVCAR-3 cells led to up-regulation of Snail protein (**Figure 4.7 A**) but not mRNA expression (**Figure 4.8)**. E-cadherin expression was not changed. Snail was localised in the nucleus only (**Figure 4.7 B**).

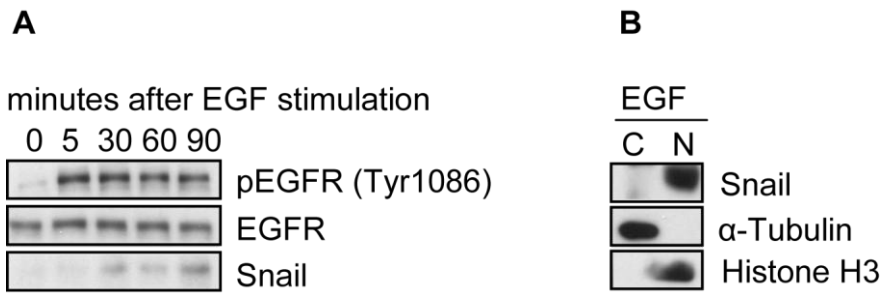

Figure 4.7 Western blot analysis of OVCAR-3 cells. (A) Cells were stimulated with EGF for 5, 30, 60 and 90 minutes before protein extraction and 1D gel electrophoresis. **(B)** Cells were stimulated with EGF for 90 minutes before cell fractionation into cytoplasmatic (C) and nuclear (N) fractions. Proteins analysed are indicated on the right. One of three independent experiments is shown.

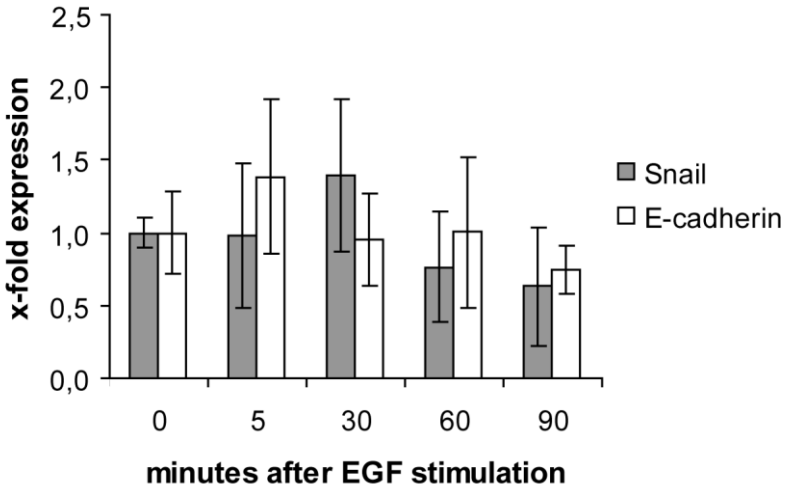

Figure 4.8 Snail and E-cadherin mRNA expression of OVCAR-3 cells. Cells were stimulated with EGF for 5, 30, 60 and 90 minutes before RNA extraction. Data were normalised to time point zero (without stimulation). The mean and standard deviation of three independent experiments, each of which was performed in duplicates, are shown.

Because EGF can activate several signalling pathways OVCAR-3 cells were pre-treated with inhibitors to EGFR (AG1478), p38 MAPK (SB203580), MEK/ERK (UO126), PI3K (LY294002), and JAKs/STATs (AG490) and Snails response to EGF stimulation was examined (**Figure 4.9**). EGF-induced Snail up-regulation required EGFR, ERK1/2 and STAT3, but not p38 MAPK and PI3K. Phosphorylation of GSK-3β and PAK1 was EGFR dependent, but due to lack of a specific inhibitor for PAK1, effects on Snail expression could not be studied. The EGFR – AKT – GSK-3β pathway is not involved in the up-

regulation of Snail in OVCAR-3 cells. The phosphorylation of HSP27 is EGFR independent**.**

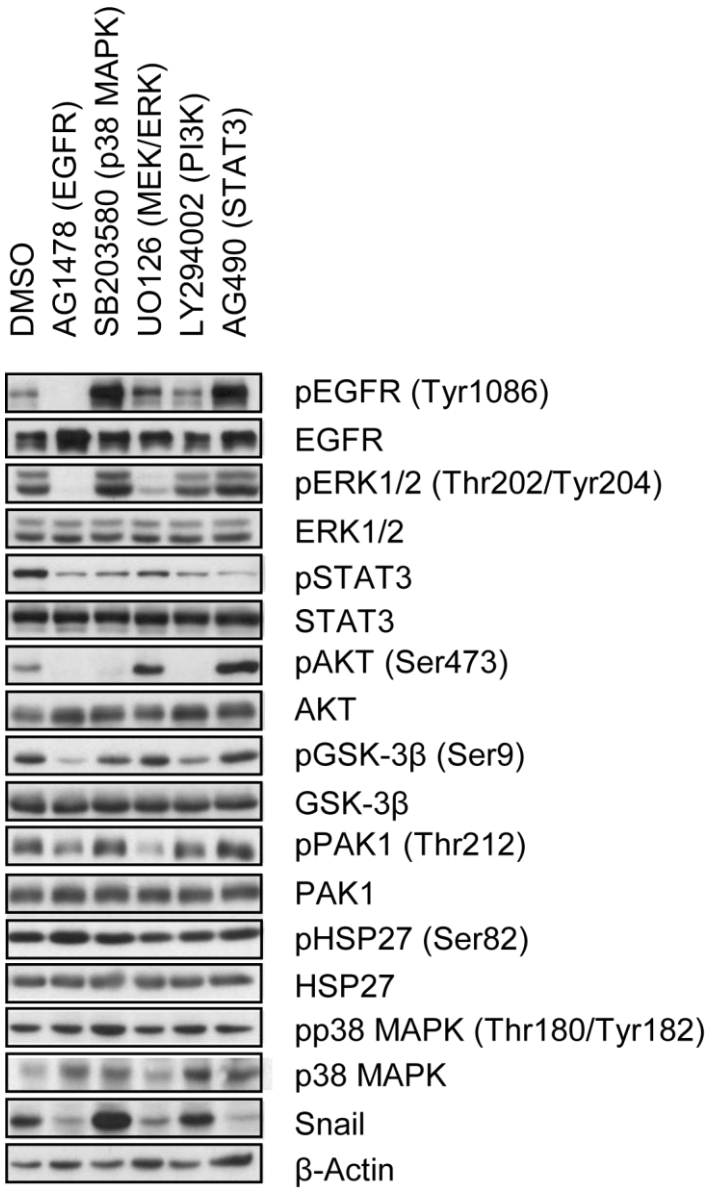

Figure 4.9 Western blot analysis of OVCAR-3 cells. Cells were incubated with the specific protein kinase inhibitors AG1478 (inhibits EGFR), SB203580 (inhibits p38 MAPK), UO126 (inhibits MEK/ERK), LY294002 (inhibits PI3K) and AG490 (inhibits JAKs/STATs) each dissolved in DMSO, respectively, 60 minutes prior to stimulation with EGF for 90 minutes. One of three independent experiments is shown.

4.2.2 Profiling of signalling proteins in primary tumours and metastasis of ovarian cancer patients using protein lysate microarrays

Using OVCAR-3 cells it was revealed that EGF leads to Snail up-regulation via ERK1/2 and STAT3. To answer the question whether EGFR activation may also be involved in

Snail regulation in primary tumours and/or metastases of ovarian cancer, full-length proteins from formalin-fixed and paraffin embedded tissues were extracted and applied on protein lysate microarrays.

After having demonstrated the specificities of all 25 antibodies (**see chapter 4.1.2.1, Figure 4.5**), protein lysate microarray analysis with protein extracts from 25 FFPE primary tumours and 24 corresponding metastases was performed in order to correlate Snail expression to abundances of a number of signalling and cell adhesion proteins.

Expression of 13 of the 25 proteins analysed, correlated statistically significant in primary tumours and metastases (**Table 4-1**), e.g. where these proteins were up-regulated (or down-regulated) in primary tumours, they were also up-regulated (or down-regulated) in metastases.

In cluster analysis (**Figure 4.10**) a clear difference of protein expression profiles of primary tumours and metastases was observed.

For analysis of the relationship between expression of Snail and several EMT regulators and signal transduction proteins, spearman rank correlation test was done (**Table 4-2**). A significant correlation was observed between Snail and the active forms of EGFR, p38 MAPK, PAK1, ERK1/2 and HSP27 and the expression of total PTEN and GSK-3β. No statistical relationship was found between Snail up-regulation and E-cadherin and Cytokeratin 18 down-regulation and Snail up-regulation and Vimentin up-regulation. In metastases no correlation between Snail and the other proteins was seen.

Table 4-1 Correlation (Spearman rank correlation test) of protein expression in primary tumours and metastases.

Protein	*rho*	*P*
pAKT (Ser473)	**0.750**	**<0.001***
AKT	**0.750**	**<0.001***
Cytokeratin18	0.550	0.005
E-cadherin	0.346	0.098
pEGFR (Tyr1086)	0.394	0.057
EGFR	**0.68**	**<0.001***
pERα (Ser118)	0.550	0.005
ERα	**0.799**	**<0.001***
pERK1/2 (Thr202/Tyr204)	0.048	0.825
ERK1/2	**0.701**	**<0.001***
pGSK-3β (Ser9)	**0.844**	**<0.001***
GSK-3β	**0.67**	**<0.001***
pHER2 (Tyr1248)	**0.659**	**<0.001***
HER2	**0.683**	**<0.001***
pHSP27 (Ser82)	**0.614**	**0.001***
HSP27	0.571	0.004
pp38 (Thr180/Tyr182)	**0.742**	**<0.001***
p38	0.479	0.021
pPAK1 (Thr212)	0.420	0.041
PAK1	0.583	0.003
PTEN	**0.744**	**<0.001***
pSTAT3 (Tyr705)	0.480	0.018
STAT3	**0.731**	**<0.001***
Snail	0.477	0.018
Vimentin	0.522	0.009

* statistically significant at a Bonferroni-adjusted level of significance of 0.0018.

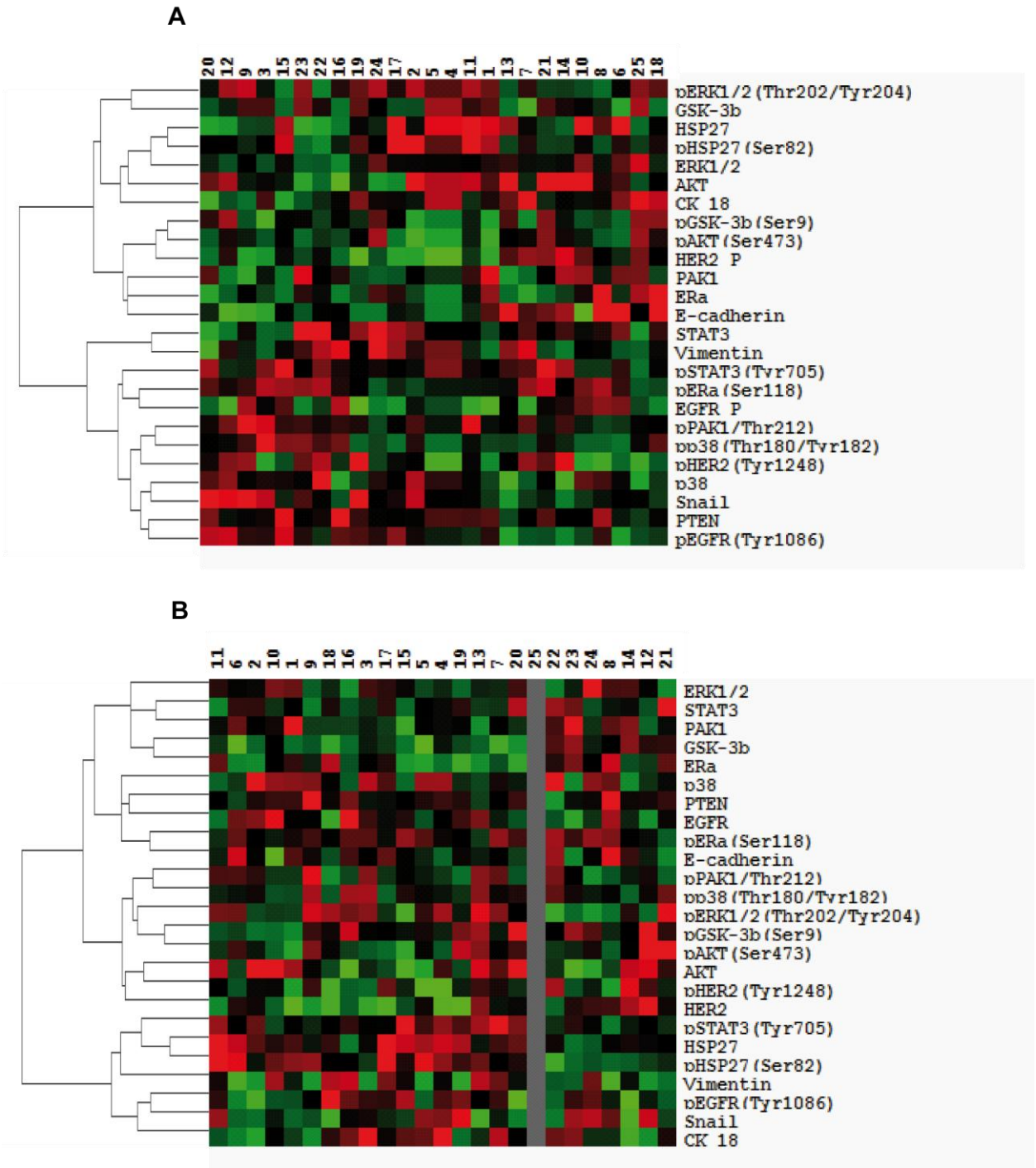

Figure 4.10 Unsupervised hierarchical clustering image map. Twenty-five proteins were used in the analysis of 25 ovarian carcinoma primary tumours **(A)** and corresponding metastases **(B)**. The patients are numbered in the horizontal axis and the protein expression is indicated on the vertical axis. Higher protein expression levels are represented in red, lower levels in green. For patient No. 25 protein extraction from metastasis was not successful (indicated in grey).

Table 4-2 Correlation (spearman rank correlation test) between different EMT regulators and signal transduction proteins and Snail in primary tumours and metastases

		Primary tumours		Metastases	
		Rho	***P***	***Rho***	***P***
Snail	E-cadherin	-0.0375	0.8586	0.4243	0.0388
Snail	Cytokeratin 18	-0.1117	0.5951	0.4381	0.0323
Snail	Vimentin	0.3138	0.1267	0.1176	0.5842
Snail	**pEGFR (Tyr 1086)**	**0.8354**	**<0.0001***	0.6135	0.0014
Snail	EGFR	0.4451	0.0258	0.2717	0.1991
Snail	pHER2 (Tyr1248)	0.5295	0.0065	0.3195	0.1281
Snail	HER2	0.3146	0.1256	0.3736	0.0721
Snail	pERα (Ser118)	0.5914	0.0018	0.6112	0.0015
Snail	ERα	0.3795	0.0613	0.5355	0.0070
Snail	pAKT (Ser473)	0.5539	0.0041	0.3221	0.1248
Snail	AKT	0.2092	0.3156	-0.1833	0.3912
Snail	**PTEN**	**0.7194**	**0.0001***	0.6046	0.0018
Snail	pGSK3β (Ser9)	0.4965	0.0116	0.4720	0.0199
Snail	**GSK3β**	**0.7068**	**0.0001***	0.5970	0.0021
Snail	**pp38 (Thr180/Tyr182)**	**0.6809**	**0.0002***	0.5248	0.0085
Snail	**p38**	**0.6778**	**0.0002***	0.3882	0.0609
Snail	**pPAK1 (Thr212)**	**0.7335**	**<0.0001***	0.4117	0.0456
Snail	PAK1	0.4968	0.0115	0.5492	0.0054
Snail	**pERK 1/2 (Thr202/Tyr204)**	**0.6729**	**0.0002***	0.0052	0.9807
Snail	ERK	0.5408	0.0052	0.5631	0.0042
Snail	**pHSP27 (Ser82)**	**0.6452**	**0.0005***	0.1951	0.3609
Snail	HSP27	-0.0869	0.6796	0.3452	0.0986
Snail	pSTAT3 (Tyr705)	0.4272	0.0332	0.3447	0.0991
Snail	STAT3	0.3089	0.1330	0.1923	0.3679

*Significant at a Bonferroni-adjusted level of significance $p<0.0007$

4.2.3 Signalling pathways and survival of ovarian carcinoma patients

The results of the Cox-Hazard-Regression analyses are summarized in **Table 4-3**. The first two models show the results of the univariable analyses of Snail and E-cadherin in primary tumours, revealing that there is no evidence for a single significant impact of Snail on the risk of death (p-value = 0.610) and E-cadherin (p=0.097). The 95%-confidence intervals of the Hazard Ratio estimations show that the factorial risk-increment in the underlying population have to be expected in a range of 0.17 to 2.83 (Snail) and 0.11 to 1.20 (E-cadherin) with a probability of 95%. If there was a significant

impact of Snail or E-cadherin according to survival, the critical value of one would not be included in these intervals.

As the biological pathway suggested relations between Snail and pp38 MAPK (Thr180/Tyr182), an interaction term was included in the model which is displayed by model 3 (**Table 4-3**). In addition to each risk factor's contribution to the Hazard Ratio this interaction term incorporates change of risk by different parameter constellations. All coefficient p-values are lower than the 5% significance level. Thus there is some evidence that an individual's hazard can be explained by Snail, pp38 MAPK (Thr180/Tyr182) and their interaction. To assess the Hazard Ratio of an individual in comparison to a person with an expression of Snail=0 and pp38 MAPK (Thr180/Tyr182)=0 (reference person), one has to solve the equation Hazard Ratio =exp(- 4.78 * Snail - 7.00 * pp38(Thr180/Tyr182) + 12.81 * Snail * pp38 MAPK (Thr180/Tyr182). To facilitate the interpretation of this relationship the Hazard Ratio is illustrated in (**Figure 4.11**). The illustration reveals that high values of both, Snail and pp38 MAPK (Thr180/Tyr182) led to a high risk for death. A low value in one factor combined with a high value in the other factor will produce a low likelihood ratio.

Table 4-3 Cox Proportional-Hazard-Models for the factors Snail, E-cadherin and interaction: Snail and pp38 MAPK (Thr180/Tyr182) in primary tumours.

Model				95%-CI Hazard Ratio		
		coef	Hazard Ratio	lower	upper	p-value
1	Snail	-0.36	0.70	0.17	2.83	0.610
2	E-cadherin	-1.02	0.36	0.107	1.20	0.097
3	Snail	-4.78	8.42e-03	1.4e-04	0.48	0.021
	pp38(Thr180/Tyr182)	-7.00	9.12e-04	3.3e-06	0.25	0.015
	Interaction	12.8	3.66e+05	15.9	8.45e+09	0.012

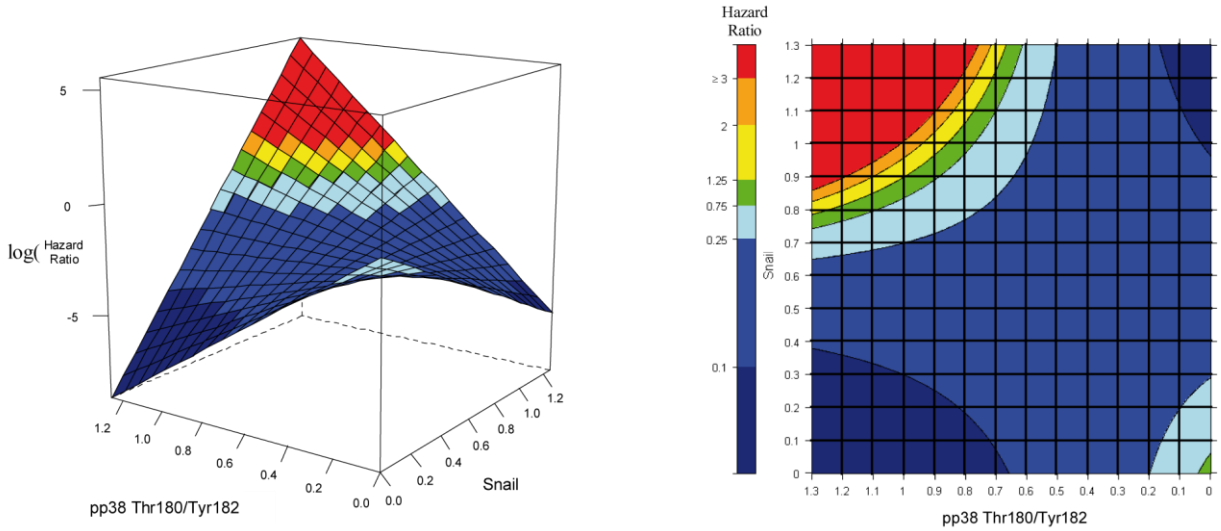

Figure 4.11 Estimated Hazard Ratio (regarding the reference risk of death associated with a zero expression of both Snail and pp38 MAPK (Thr180/Tyr182)) of death in dependence on interaction of Snail and pp38 MAPK (Thr180/Tyr182). Colours indicate the magnitude of the Hazard Ratio which is displayed in the appended legend. Please note that due to optical agreement of the graphs the scaling is not arranged in the classical fashion of ascending values from the left to the right. (**A**) Wireframe graphic; (**B**) Contour plot.

5 Discussion

The main goal of my studies was to understand in more detail the mechanisms regulating epithelial tumour markers by mapping the transcriptional repressor Snail and the cell adhesion proteins E-cadherin and Cytokeratin 18 and potential signal transduction pathways, in cell lines and tissues from endometrial and ovarian cancers.

E-cadherin is one of the caretakers of the epithelial phenotype and is responsible for stable cell-cell contacts and adherence junctions. There is a direct correlation between lack of E-cadherin and loss of the epithelial phenotype. Loss of E-cadherin has also been described in relation with invasive behaviour of tumour cells and a higher tumour grade. The zinc finger transcriptional repressor Snail binds to two E2 boxes that are located proximal to the transcriptional start site of the E-cadherin gene (Batlle et al., 2000; Cano et al., 2000).

5.1 Regulation of the E-cadherin repressor Snail in endometrial carcinoma

In the endometrial carcinoma cell line Ishikawa EGF stimulation led to an activation of the ERK1/2 pathway and Snail mRNA and protein levels were increased. In contrast in Ishikawa ER- cells EGF led to activation of AKT and ERK1/2 pathways, what resulted in up-regulation of Snail protein but not Snail mRNA levels (**Figure 5.1 A, B**). Inhibition of EGFR prevented in both cell lines phosphorylation of AKT, leading to the conclusion that AKT is a downstream target of EGFR. But only in Ishikawa ER- cells inhibition of AKT resulted in Snail down-regulation. The increased phosphorylation of EGFR after inhibition of p38 MAPK led to the suggestion that the cells try to compensate the inhibition by enhanced activation of EGFR. The elevated expression of EGFR may result from reduced internalisation after ligand binding (Wiley, 2003). No influence of the p38 MAPK pathway on Snail protein level was seen in Ishikawa and Ishikawa ER- cells. However, in the Ishikawa and Ishikawa ER- cell lines GSK-3β phosphorylations were not influenced by EGF. Lu et al. already reported no significant change in the level of phosphorylated GSK-3β in response to EGF in A431 cells (Lu et al., 2003). In Ishikawa cells no change in HSP27 phosphorylation was seen after EGF stimulation, in Ishikawa ER- cells phosphorylated HSP27 levels were reduced after inhibition of EGFR, indicating that HSP27 is a downstream target of EGFR in these

cells. But due to lack of specific inhibitors for HSP27, effects on Snail expression could not be examined.

It should be kept in mind, however, that a single cell line may not be representative for a complex disease like cancer, which involves many different subtypes and a close interaction between tumour and stromal cells. In this study each of the 17 tumours showed a different protein expression profile, indicating a complex, patient-specific regulation of Snail in human endometrial cancers and emphasising the need for individualized therapy regimens targeting several signalling pathways at the same time.

Previous studies reported that decreased E-cadherin expression correlated with loss of differentiation and depth of myometrial invasion in human endometrial carcinoma (Sakuragi et al., 1994). Blechschmidt et al. already found that abnormal E-cadherin expression correlated with positive Snail immunoreactivity in metastases of endometrial carcinomas and suggested that Snail might play a role in tumour dedifferentiation and progression (Blechschmidt et al., 2007). With the protein microarray data reported in this study the inverse correlation between Snail and E-cadherin expression could be confirmed and additional an inverse correlation between Snail and Cytokeratin 18 was found.

The results of this study revealed a correlation between EGFR signalling with increased abundances of the Snail protein, possibly regulated by the p38 MAPK, ERK1/2 and the AKT/GSK-3β pathways, and down-regulation of E-cadherin and Cytokeratin 18 in primary endometrial tumours. In the primary tumours one large cluster (group1) including Snail and the activated forms of EGFR, GSK-3β, p38 MAPK, AKT, HER2, PAK1 and ERK1/2 was seen. Zohn et al. reported that p38 MAPK activation *in vivo* is required for down-regulation of E-cadherin protein during gastrulation in mice. p38 MAPK regulates E-cadherin expression independently of the transcriptional repressor Snail (Zohn et al., 2006). In the protein lysate study an inverse correlation between p38 and E-cadherin expression was seen. In human dermal fibroblasts EGF treatment resulted in activation of p38 MAPK (Yamane et al., 2007). In the primary endometrial carcinoma tissues expression of Snail clustered very close to the phosphorylated forms of EGFR, p38 MAPK, AKT and GSK-3β, indicating that these pathways may play an important role in this disease.

Inactivation of PTEN is the most common genetic defect in endometrioid endometrial carcinoma (Hecht & Mutter, 2006; Inoue, 2001; Lax, 2004; Liu, 2007;

Matias-Guiu et al., 2001), which results in increased activation of the PI3K/AKT pathway. Therefore it was unexpected, that in this study a close clustering of the active, phosphorylated form of AKT and PTEN was observed. In human T cell acute lymphoblastic leukaemia cells it was shown, that PTEN inactivation is not always synonymous with reduced PTEN protein expression. Silva et al. supposed that posttranslational mechanisms down-regulate PTEN activity, but not protein expression, and promote PI3K/AKT hyperactivation (Silva et al., 2008). In contrast, the mechanism described by Escrivà et al., that Snail represses the promoter activity of PTEN (Escriva et al., 2008), seems not to play a role in endometrial cancer.

In addition an inverse clustering between Snail and HSP27 was found in the primary tumours, what is in line with Vidyasagar et al. who reported that HSP27 may modulate EMT through up-regulation of E-cadherin expression by down-regulating Snail expression (Vidyasagar et al., 2008).

In this study the ERK1/2 pathway was confirmed to be involved in the activation of Snail in both Ishikawa cell lines and in primary endometrial tumours, as reported by others using promoter analysis (Barbera et al., 2004). It was interesting to see in cluster analysis of the endometrial primary tumours a close association of the active forms of PAK1 and ERK1/2. Sundberg-Smith et al. reported that adhesion signalling induces a direct association between ERK and PAK1. They showed that ERK2 phosphorylates PAK1 on Threonine 212 *in vitro* in rat smooth muscle cells, which may provide negative feedback inhibition of ERK signalling (Sundberg-Smith et al., 2005). PAK1 was also reported to phosphorylate Snail and to promote its localisation into the nucleus in human breast cancer cell lines (Yang et al., 2005). In this study, however, no EGF related correlation between PAK1 and Snail in the cell lines was seen, but the active form of PAK1 clustered close to Snail in primary tumours.

In contrast to Julien et al., who found that NF-κB expression induces Snail expression in LX-B cells (Julien et al., 2007), we could not find a positive correlation between NF-κBp65 and Snail. But as already described by Saegusa et al. in endometrial carcinomas, positive correlation between NF-κBp65 and E-cadherin and an inverse correlation between NF-κBp65 and Snail was found (Saegusa et al., 2007).

HER2/neu over-expression occurs in progression and dedifferentiation in about 15 to 20 % of endometrial cancers and often correlates with a higher tumour grade and a poor survival (Rolitsky et al., 1999; Treeck et al., 2003). Accumulating evidence suggests that activated HER2 leads to the onset of intracellular signalling events

involving ERK (Bourguignon et al., 2007). In the current study a correlation between the phosphorylated forms of HER2, PAK1 and ERK1/2 was found, suggesting that this pathway may also be involved in the pathogenesis of human endometrioid endometrial carcinoma. Possibly HER2 is activated by heterodimerisation with EGFR, as the phosphorylated forms of HER2 and EGFR cluster together in one group.

Park et al. described that oestrogen regulates Snail and Slug mediated E-cadherin repression (Park et al., 2008). In contrast, in this study Snail and ERα show an inverse correlation, what fits to Dhasarathy et al., who described that ERα dependent expression of MTA-3 leads to Snail repression and in turn Snail expression leads to loss of ERα expression at mRNA and protein levels (Dhasarathy et al., 2007).

Snail protein and mRNA levels did not correlate in the primary tumour tissues, as already seen in Ishikawa ER- cells, suggesting posttranslational mechanisms taking part in regulation of Snail protein expression.

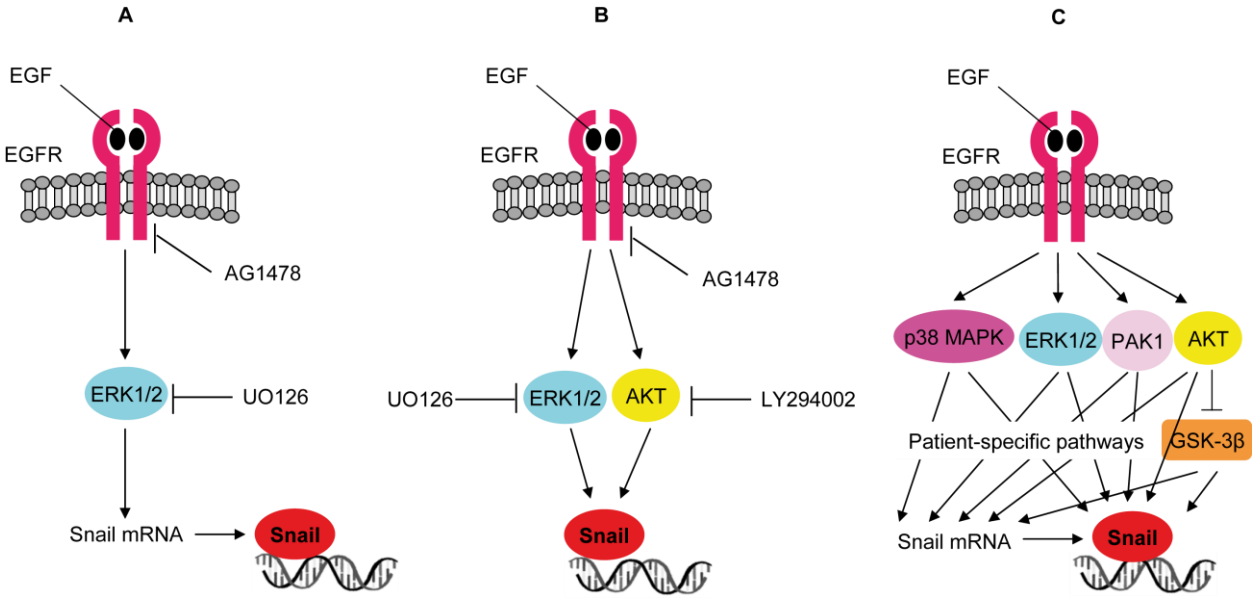

Figure 5.1 Proposed model for EGFR related signal transduction pathways regulating Snail in Ishikawa cells (A), Ishikawa ER- cells (B) and primary human endometrial tumours (C). (**A**) In Ishikawa cells, EGF stimulation leads to up-regulation of Snail mRNA and protein expression via ERK1/2. (**B**) In Ishikawa ER- cells, EGF leads to Snail protein over-expression, via ERK1/2 and AKT pathways. (**C**) In primary tumours of the endometrium, EGF stimulates pathways leading to Snail's localisation into the nucleus as well as pathways involved in reduced degradation of the protein. Snail shuttles into the nucleus after phosphorylation on Serine 246 by an unknown pathway, possibly with involvement of p38 MAPK, AKT, ERK1/2 and PAK1, respectively. After phosphorylation of GSK-3β on Serine 9 by AKT, GSK-3β is unable to phosphorylate and tag Snail for ubiquitination and proteasomal degradation. Our data clearly demonstrate that cell lines cannot fully reflect the activation status of signalling pathways in primary cancers.

5.2 Regulation of the E-cadherin repressor Snail in ovarian carcinoma

EGFR over-expression correlates with tumour progression, metastasis and poor clinical outcome in a variety of carcinomas, but the underlying mechanisms are poorly understood (Lu et al., 2003). Sewell et al. reported that inhibition of EGFR by Iressa (ZD 1839) inhibited growth of ovarian cancer xenograft models (Sewell et al., 2002). In this study the EGFR – ERK1/2 – STAT3 pathway could be revealed in OVCAR-3 cells, being responsible for up-regulation of Snail protein expression (**Figure 5.2 A**). To confirm the results in human ovarian cancer tissue, expression of 25 proteins was analysed by protein lysate microarrays in a series of 25 primary ovarian carcinomas and their corresponding metastases and the activation of EGFR could be approved correlating with Snail expression.

Unlike Lu et al., who reported increased Snail mRNA levels after EGF stimulation in A431 cells (Lu et al., 2003), but in line with Lee et al., who found a correlation between Snail and EGFR activation in the cervical cancer cell line CaSki (Lee et al., 2008), in OVCAR-3 cells only increased Snail protein, but not mRNA levels could be detected after EGF stimulation. Lo et al. reported that EGFR cooperates with STAT3 to induce Twist expression in MDA-MB-468 breast cancer cells and primary breast carcinomas (Lo et al., 2007); in contrast to these findings in OVCAR-3 cells no effects of EGF on Twist expression were found (data not shown). Increased Snail expression had no impact on E-cadherin levels, suggesting that up-regulation of Snail after EGF stimulation may not be sufficient for induction of EMT.

Because a single cell line is not representative for a complex disease like cancer, analysis of primary tumours and metastases was included in the study. A statistically significant correlation of the active forms of EGFR, p38 MAPK, PAK1 and ERK1/2 with Snail have been found in primary tumours of ovarian carcinoma (**Figure 5.2 B**). All these pathways have been found previously to play a role in the regulation of Snail. In cervical cancer cells it was shown recently that EGFR inactivates GSK-3β, what results in nuclear accumulation of Snail (Lee et al., 2008). p38 MAPK and ERK1/2 have been shown to act as mediators of Snail and Slug expression after UV exposure in keratinocytes (Hudson et al., 2007). PAK1 promotes Snail's nuclear accumulation and consequently its repressor activity in human breast cancer (Yang et al., 2005). No significant correlation between Snail and the inactive form of GSK-3β (Schlessinger &

Hall, 2004) and the active form of AKT (Grille et al., 2003) was observed, but a statistically significant correlation between PTEN and Snail was found, suggesting that in ovarian cancer primary tumours, PTEN represses activation of the PI3K/AKT pathways and consequently GSK-3β is not phosphorylated on Serine 9 and therefore does not contribute to Snail's nuclear accumulation.

An interesting observation made in this study was that each of the ovarian cancer patients showed different protein expression profiles (**Figure 4.10**) as already described by Sheehan et al. (Sheehan et al., 2005). Thus protein profiling with reverse phase protein microarrays using FFPE samples (in our study) or frozen samples (Sheehan et al., 2005) show the same results: signalling pathways in ovarian cancer patients are complex and patient-specific. In tumour progression not only signalling cascades of the tumour cell itself, but also pathways activated in cells of the tumour microenvironment play an important role (Hanahan & Weinberg, 2000). Therefore, profiling and classification of cell signalling pathways in tumour tissues would be an important addition to the pathological reporting of cancer tissues, for diagnosis, response analysis and patient tailored therapy (Gulmann et al., 2006; Sheehan et al., 2005).

Because metastases derive from the primary tumours, it is expected that their molecular profiles are similar to those of primary cancers. Metastasis is a multistep process including detachment from the primary tumour, invasion into surrounding connective tissue, intravasation, survival in lymph and blood vessels, extravasation and settlement at target organs. This process requires the survival of clones that are able to obtain genetic or epigenetic changes, necessary for metastasis. It is known that most cancers, due to their genetic instabilities, produce heterogeneous clones while they grow at primary sites and so eventually produce metastasis competent clones (Kim et al., 2008). As already described by Belluco et al. for colon cancer, by Kim et al. for gastric cancer and by Sheehan et al. for ovarian cancer (Belluco et al., 2005; Kim et al., 2008; Sheehan et al., 2005), in this study protein expression profiles of primary tumours and metastasis also showed clear differences. The tissue microenvironment of metastasis is different from that of the primary tumour, so Wulfkuhle et al. already expected that phosphorylation events in metastatic cells differ significantly from those of primary tumour cells. This indicates the need to include profiling of metastatic tissue for treatment selection, because the effective treatment of metastatic disease is the key to ultimate response (Wulfkuhle et al., 2006). Since FFPE tissues are used in all pathology

departments, this approach may be widely applied in the future in the routine setting of many hospitals.

An additional question was whether signalling pathways regulating Snail would be of prognostic value. A statistical significant correlation between the interaction of the phosphorylated form of p38 MAPK (Thr180/Tyr182) and Snail (p=0.012) and reduced overall survival was found in ovarian carcinoma primary tumours. This indicates that up-regulation of Snail via p38 MAPK leads to an adverse clinical outcome for ovarian cancer patients.

No relation between the epithelial proteins E-cadherin and Cytokeratin 18, the mesenchymal marker Vimentin and Snail was observed in primary tumours. And also in the OVCAR-3 cells, increased Snail expression after EGF stimulation had no impact on E-cadherin levels. As described in **chapter 1.4.2.1**, Peinado et al. showed a model that Snail and Sip1 play a role in the induction of tumour progression and that other repressors, Slug and E-47, are responsible for maintaining the mesenchymal phenotype (Peinado et al., 2004). In the near future, when specific antibodies against other E-cadherin repressors are available, it would be interesting to include analysis of these proteins. Snail may also function as a regulator of cell cycle and apoptosis in these cells, as reported by Vega et al. in MDCK cells (Vega et al., 2004).

In metastases (**Figure 5.2 C**) we did not see a statistically significant correlation between Snail and the proteins analysed, suggesting that other signalling pathways, like TGFβ or Wnt, which have not been analyzed in this study, contribute to the regulation of Snail.

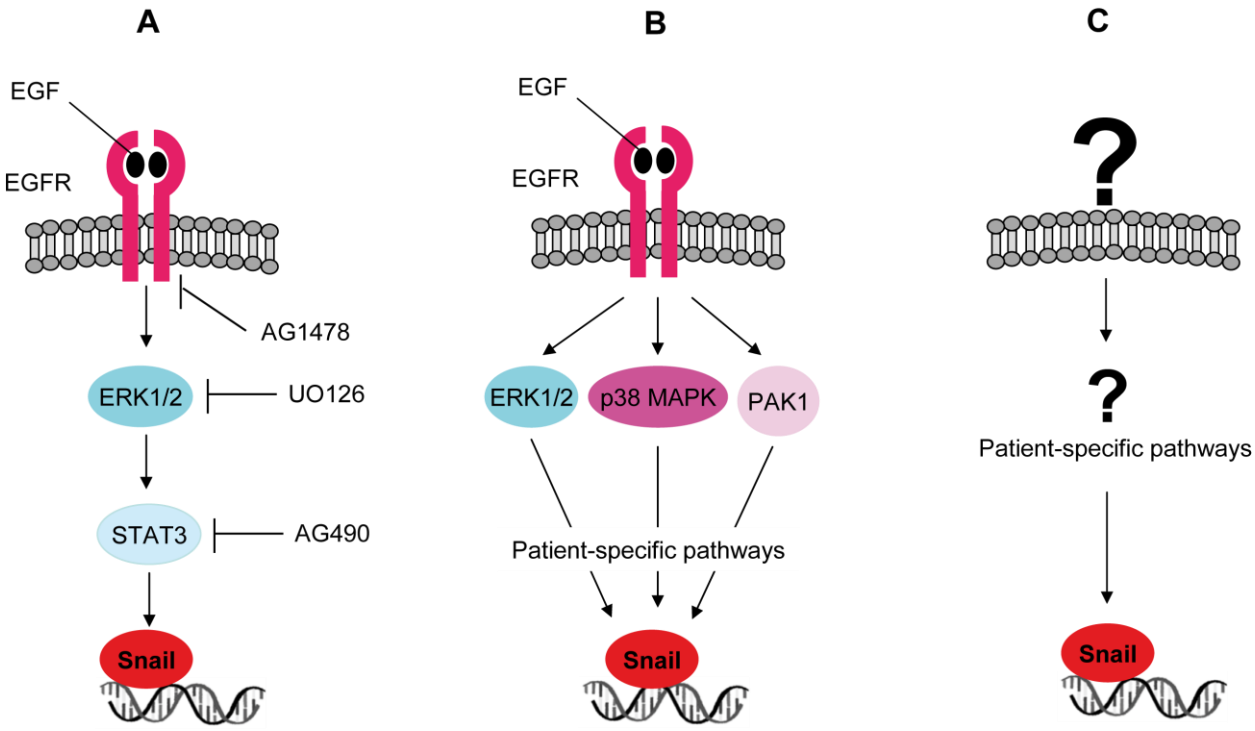

Figure 5.2 Proposed model for EGFR related signal transduction pathways regulating Snail in OVCAR-3 cells (A), primary tumours (B), and metastases (C) of ovarian carcinoma. (**A**) In OVCAR-3 cells the ERK1/2 – STAT3 pathways participates in up-regulation of Snail after EGF stimulation. In primary tumours (**B**) possibly the EGFR, p38 MAPK, ERK1/2 and PAK1 pathways are involved in Snail up-regulation. In metastases (**C**) none of the profiled pathways are involved in the regulation of Snail.

5.3 Protein lysate microarrays and their impact on individualized cancer diagnosis and therapy

Dissociation of cancer cells from the primary tumour is a crucial step for invasion and metastasis. Transmission of intracellular signals by reversible phosphorylation and dephosphorylation is a common molecular mechanism in this step. Therefore investigation in quantification of the phosphorylation state of a protein is of high interest (Becker et al., 2008). Until recently, it was not possible to molecular profile protein networks critical for this process using typical clinical samples, e.g. formalin-fixed tissues. Protein lysate microarrays are an excellent tool for quantification of protein expression in tumour tissues because they use internal standard curves, are sensitive and require low amounts of samples (Becker et al., 2008; Chan et al., 2004; Espina et al., 2003; Grubb et al., 2003; Liotta et al., 2003; Paweletz et al., 2001; Tibes et al.,

2006). Formalin fixed and paraffin embedded tissues have been used for decades for routine histopathological examinations. Recently it was demonstrated that extraction of non-degraded proteins of these tissues is possible for quantitative analysis using protein microarrays. Becker et al. showed that protein yields, protein expression patterns and immunoreactivity between formalin-fixed and unfixed cells do not differ. Comparison of frozen and formalin-fixed tissues showed a slight difference in protein patterns, but immunoreactivity was very similar (Becker et al., 2007b).

A general observation I made while studying expression of phosphorylated proteins in tissues was that expression of phosphorylated and non-phosphorylated forms of a protein do not necessarily correlate. Different affinities of the antibodies for each form may explain at least in part this observation. In line with this observation is the fact that we could detect phosphorylated EGFR (Tyr1086) in the breast cancer cell line MDA-MB-435s after stimulation with EGF, but EGFR expression in this cell line could not be proven (data not shown).

Results obtained from immunohistochemistry do not necessarily correlate with results from protein lysate studies, due to the fact that evaluation of immunohistochemistry stainings is, at least in part, dependent from the observer and different antigen demasking procedures are used. But in immunohistochemistry staining, the localisation of a protein can be determined. One limitation of the detection by chemiluminescence, as used for these studies, is the inability to multiplex proteomic-based endpoints, as each array can only report one analyte endpoint. Therefore Calvert et al. established a two-dye fluorescent detection system using secondary antibodies that have been labelled with fluorochromes that can be detected simultaneously at 700 nm and 800 nm wavelength, respectively (Calvert et al., 2004). At the moment the bottleneck of this method is the lack of specific antibodies against a lot of proteins and also the fact that for multiplex detection, antibodies from different species are needed.

Protein lysate microarrays from formalin-fixed tissues are a new method with potential for clinical implementation, without the need for changing the clinical workflow, such as formalin-fixation for standard histopathological examinations. As soon as more antibodies are available, mapping of tumour cell protein networks will be critical for realising the promise of personalised medicine. In the near future, protein lysate microarrays may be used in the clinic for diagnosis, response prediction and selection of patient-tailored therapies.

5.4 Conclusions

In this thesis I could show, that EGFR signalling plays a role for Snail expression both in the endometrial cancer cell lines Ishikawa and Ishikawa ER- and the ovarian cancer cell line OVCAR-3 and primary tumours of endometrial and ovarian cancers. The pathways found, being involved in the regulation of Snail differed in cell lines and tumours and also between the two cancer types. So that I can conclude, that signalling pathways downstream of EGFR are cancer type specific and that cell lines do not necessarily reflect a complex disease like cancer.

In endometrial primary tumours, I found an inverse correlation between Snail and E-cadherin and also between Snail and Cytokeratin 18, so that I conclude, that Snail plays a role in EMT in these tumours. In primary tumours of ovarian carcinomas I did not find any relation between Snail and E-cadherin and Cytokeratin 18, suggesting that Snail may not play a role for EMT in these tumours and is responsible e.g. for cell cycle and apoptosis regulation. Another possibility is that Snail interacts with other transcriptional repressors, like Slug, ZEB or Twist or represses other epithelial proteins, like Occludin or Claudin. Whenever specific antibodies against these proteins are available, they should be included in the study. I found that interaction of Snail and the phosphorylated form of p38 MAPK in primary tumours leads to a significant higher risk of death in ovarian cancer patients, concluding that Snail plays a role for patient survival, independently of its function on EMT, cell cycle or apoptosis.

In my studies I applied the first time protein lysate microarrays for profiling of signal transduction pathways, possibly regulating EMT, in formalin fixed and paraffin embedded cancer tissues. First cluster analysis of protein expression revealed that protein expression profiles of primary tumours and metastases of ovarian carcinomas differed. Second cluster analysis showed that each patient has a different protein expression profile and several and different pathways are activated. Therefore in the future, novel cancer treatments should target not only one, but several signalling pathways, which are activated in the individual patient.

6 Summary

Background: Epithelial-mesenchymal transition is a cellular programme, which contributes to invasion and metastasis of carcinomas, characterised by loss of cell adhesion, repression of E-cadherin expression and increased cell motility. Snail a major regulator of EMT is one of the most prominent transcriptional repressors of the invasion and tumour suppressor E-cadherin. The signalling pathways regulating Snail protein expression in different primary human cancers however are not well understood.

Methods: Protein lysate microarray technology offers a new opportunity to measure and profile signalling pathways in cells and tissues. Recently this method was successfully introduced for the use of formalin-fixed clinical tissue samples. Formalin-fixation and paraffin embedding is the standard processing methodology for tissues practiced in pathologies world-wide.

Results: As a first cancer type, endometrial cancer was chosen. Stimulation of two endometrial carcinoma cell lines with epidermal growth factor led to an increase of Snail protein expression, but the pathways involved in this process differed. In the Ishikawa cells the ERK1/2 pathway was activated after EGF stimulation and Snail mRNA and protein levels were affected. In the Ishikawa ER- cell line AKT and ERK1/2 were activated, resulting in Snail protein, but not mRNA up-regulation. In primary human endometrioid endometrial carcinomas Snail protein expression correlated with the activated, phosphorylated form of EGFR as revealed by profiling 24 different signalling proteins using protein lysate microarrays. In addition, we observed an inverse correlation between Snail and E-cadherin protein levels in these tumours.

As a second cancer type ovarian cancer was chosen. Stimulation of the human ovarian cancer cell line OVCAR-3 revealed the EGFR – ERK1/2 – STAT3 pathway being involved in up-regulation of Snail expression. Because a single cell line cannot reflect a complex disease like cancer, 25 primary tumours and corresponding metastases were analysed for expression of 25 proteins. The phosphorylated form of EGFR correlated with Snail expression in primary tumours, but not metastases. In addition protein expression profiles of primary tumours and metastases differed and were complex and patient-specific.

Conclusions: Taken together, profiling of signalling proteins in cell lines and primary human tissues provided strong evidence that EGFR signalling is involved in Snail protein over-expression in endometrial and ovarian cancer.

7 Literature

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8 Abbreviations

Ab: Antibody

AKT: Protein kinase B

APS: Amoniumpersulfate

ATCC: American type culture collection

bHLH: basic helix-loop-helix

BSA: bovine serum albumine

cDNA: Complementary desoxyribonucleic acid

CI: confidence interval

CK: Cytokeratin

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethylsulfoxide

dNTPs: Desoxynucleotide triphosphates

dpi: dots per inch

DTT: Dithiothreitol

E2: 17ß-estradiol

ECACC: European collection of cell cultures

EDTA: Ethylenediaminetetraacetat

EGFR: Epidermal growth factor receptor

EMT: Epithelial-mesenchymal transition

ER: Oestrogen receptor

ERK: Extracellular-signal regulated kinase

FAM: 6-Carboxy-Fluorescein

FBS: Fetal bovine serum

FFPE: Formalin fixed and paraffin embedded

FIGO: Fédération Interantionale de Gynécologie et d'Obstétrique

GSK: Glycogen synthase kinase

HEPES: 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethansulfonsäure

HER : Human epidermal growth factor

HRP: horseradish

HSP: Heat shock protein

IHC: Immunohistochemistry

NF: Nuclear factor

MAPK: Mitogen activated kinases

MDCK: Madin-Darby canine kidney

MTA: metastasis associated protein

MET: Mesenchymal-epithelial transition

MP: Milk powder

NES: nucear export signal

OD: optical density

PAK: p21 activated kinase

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PI3K: Phosphoinositide 3-kinase

PKB: protein kinase B

PMSF: Phenylmethylsulfonylfluorid

PTEN: Phosphatase and tensin homologue deleted on chromosome ten

RNA: Ribonucleic acid

RT-PCR: reverse transcriptase polymerase chain polymerase

SDS: Sodium dodecyl sulphate

Ser: Serine

SIP: Smad interacting protein

STAT: Signal transducers and activators of transcription

TAMRA: Tetramethyl-6-Carboxyrhodamine

TBS: Tris buffered saline

TEMED: N, N, N', N'-Tetramethylethan-1,2-diamin

TGF: Transforming growth factor

Tif: tagged image file

UICC: International Union against cancer

WHO: World health organization

ZIP: Zrt- and Irt-like proteins