Technische Universität München

Lehrstuhl für Humanbiologie

Significance of the epithelial-mesenchymal-transition regulator Snail in human gynaecological cancers and cell lines

Kareen Blechschmidt

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende: Univ.-Prof. Dr. H. Daniel
Prüfer der Dissertation:

1. Univ.-Prof. Dr. M. Schemann
2. Priv.-Doz. Dr. K.-F. Becker

Die Dissertation wurde am 01.10.2007 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 17.01.2008 angenommen.

Acknowledgements

I want to thank PD Dr. Karl-Friedrich Becker without whom this work would not have been possible. Thank you very much for your support, advice and guidance which helped me in all the time of research for and writing of this thesis.

I am deeply grateful to Prof. Michael Schemann who kindly agreed to act as my PhD supervisor.

My appreciation goes to the head of the Institute of Pathology Prof. Heinz Höfler.

I wish to express my warm and sincere thanks to Anne Rappl, Monika Balzer, Christa Schott and Frauke Neff. Thank you so much for all your help, support, advice and encouragement throughout this work. Especially, I would like to thank you for your friendship, which means so much to me, and for a great time inside and outside of the Institute of Pathology.

I wish to thank Susi Pielnhofer, Catarina Alves, Volker Metzger, Susanne Hipp and Michaela Blöchinger for being wonderful colleagues who supported me throughout my work. Susi and Volker: I could not have thought of better office mates.

I warmly thank Judith Miske and Andre Katona for wonderful and funny “Club-Tag” events. I will always keep “Rigatoni Gorgonzola” in fond memories.

I would also like to express my gratitude to all the other colleagues from the Institute of Pathology who supported me in my research work. I want to thank them for all their help, interest and valuable hints.

During this work I have collaborated with esteemed colleagues from the 1st Department of Obstetrics and Gynaecology of the LMU Munich, and I wish to express my sincere thanks to PD Dr. Udo Jeschke, Dr. Ioannis Mylonas, Sandra Schulze and Christina Kuhn. Thank you very much for your warm welcome and a wonderful and effective collaboration.

Many thanks to Carsten Zimmermann without whom I would have freaked out by formatting this manuscript. Thank you very much for your help and your patience.

Finally, I wish to express my love and gratitude to my whole family. Thank you so much, Mama, Papa, Marianne, Omi and Ute, for all your love, support, interest and for being right there for me whenever I need you!

TABLE OF CONTENTS

1 INTRODUCTION

1.1 ENDOMETRIAL CANCER

In developed countries, endometrial cancer is the most common gynaecological cancer and the fourth most common malignancy in women after breast, lung, and colorectal cancers [97, 125]. Endometrial cancer in general has a good prognosis, with 5-year survival rates around 80% in the developed world [125]. However, patients with deep myometrial invasion, poor tumor differentiation and advanced disease have a poorer prognosis and are at higher risk for disease recurrence [67, 68, 86]. Endometrial carcinomas can be divided on clinical and prognostic grounds into two main subgroups [16, 75, 80]. The first group consists of estrogen-dependent tumors that are typically well-differentiated endometrioid carcinomas (EECs). EECs usually develop in pre- and perimenopausal women and coexist with or are preceded by complex and atypical endometrial hyperplasia. The second group are the high-grade non-endometrioid carcinomas (NEECs) – mainly papillary serous and clear cell carcinomas – that occur predominantly in postmenopausal women. They are aggressive tumors, independent of estrogen exposure, occasionally arising in endometrial polyps or from precancerous lesions that develop in atrophic endometrium. Although risk factors and molecular pathogenesis are different for EECs and NEECs [3, 75, 80, 122] recent immunohistochemical analyses have indicated that decreased expression of the Ca^{2+}-dependent cell adhesion molecule E-cadherin on the tumor cellular membrane is correlated with known adverse prognostic features, e.g. tumor dedifferentiation and deep myometrial invasion, and lower overall survival in both types of endometrial cancer [53, 69, 76, 123, 124].

1.2 OVARIAN CANCER

Epithelial ovarian cancer is the second most common gynaecological cancer and the leading cause of death among female genital malignancies in the developed world [97, 125]. It is widely accepted that a strong family history of ovarian and breast cancer as well as nulliparity is associated with an increased risk of ovarian cancer, whereas oral contraceptive use, pregnancy (regardless of gestational length or outcome), and lactation are associated with a reduced risk [20, 153]. More than two thirds of patients with ovarian cancer are diagnosed with advanced-stage disease, because ovarian cancer is often asymptomatic in its early stages [95]. The degree of peritoneal dissemination is related to the poor prognosis in patients with advanced-stage ovarian cancer. The molecular mechanisms that allow ovarian cancer cells to detach from the primary tumor, invade the peritoneal surfaces, and regrow at this site are not yet well understood. Previous studies have shown that reduced expression of the cell adhesion molecule E-cadherin in ovarian cancer is associated with the invasive phenotype, advancing tumor stage, lower 5 year survival rate and poor recurrence-free survival [36, 58, 78, 147] but not much is known about the underlying mechanisms of E-cadherin down-regulation in ovarian cancer cells.

1.3 PLACENTATION

A natural model for migration and invasion which shows many similarities to the growth of cancer cells, is placental development during embryo implantation. This highly complex but coordinated process relies fundamentally upon the differentiation and organisation of different trophoblastic cell populations with specific functions [28, 73]. While villous trophoblast cells remain in the fetal compartment and undergo terminal differentiation and fusion to form the multinucleate syncytiotrophoblast, extravillous trophoblast cells extensively infiltrate the endomyometrium, anchoring the placenta and modifying maternal spiral arteries [70, 108]. Aberrant differentiation and resulting shallow interstitial and vascular invasion of extravillous cytotrophoblast cells are thought to be associated with complications of pregnancy such as preeclampsia (PE), intrauterine growth restriction (IUGR) and in severe cases second trimester miscarriage [107].

Expression of the cell adhesion molecule E-cadherin in normal placentas was shown to be high in cytotrophoblast stem cells of anchoring villi and is considered to be down-regulated or absent in invading extravillous trophoblast cells in normal pregnancies [4, 165]. Floridon *et al.* described a temporary down-regulation of E-cadherin expression in extravillous trophoblast cells during interstitial and endovascular invasion with the potential to restore E-cadherin expression after completion of migration [37]. It was further shown that E-cadherin expression was up-regulated in interstitial and vascular extravillous trophoblasts in placental bed biopsies from patients with preeclampsia [164].

1.3.1 Preeclampsia

Preeclampsia, i.e. hypertension and proteinuria, in pregnancy is amongst the leading causes for maternal and perinatal morbidity and mortality [87, 115, 133, 150]. The etiologic factors causing this disease are still not clear although different risk factors for preeclampsia are known including primiparity, obesity, (family) history of preeclampsia, multiple pregnancies, and chronic medical conditions such as long-term hypertension or diabetes [33]. About 6% to 8% of pregnancies are complicated by preeclampsia and women with early onset disease show a severer form of preeclampsia than women with later onset disease [87, 133]. Preeclampsia can not be prevented and treatment of preeclampsia patients often requires immediate delivery, with consequent iatrogenic fetal prematurity [115].

1.3.2 HELLP Syndrome

As described by Weinstein in 1982 [151], HELLP syndrome is a variant of severe preeclampsia recognized as a serious, life-threatening disease characterized by **h**emolysis, **e**levated **l**iver enzymes, and **l**ow **p**latelets. The incidence of HELLP syndrome is reported as being 0.17% to 0.85% of all live births with a reported perinatal death rate ranging from 7.4% to 20% [116, 132]. The associated liver disease may be severe and in rare cases rupture of the liver can require maternal liver transplantation [128]. The pathogenesis of the HELLP syndrome is still quite unclear. It is thought that inadequate placental development is followed by placental ischemia, leading to endothelial damage with release of vasoconstrictors into the maternal circulation [65]. Multiple genetic, immunologic and coagulative disorders

seem to contribute to the endothelial damage [65]. As for preeclampsia, treatment of HELLP syndrome patients often requires immediate delivery for maternal or fetal indications, with the neonate being exposed to all risks as a preterm born [115, 116].

1.4 EPITHELIAL-MESENCHYMAL-TRANSITION (EMT)

Predominantly epithelial-mesenchymal-transition (EMT) is a key process in normal embryonic development during gastrulation movements and neural crest formation. It can be defined as a process that produces complete loss of epithelial traits by the former epithelial cells accompanied by total acquisition of mesenchymal characteristics (**Figure 1-1**) [49, 50, 140, 141]. The cells become able to dissociate from the epithelial tissue from which they originate without undergoing apoptosis or anoikis. Instead, these cells have the ability to invade and migrate through the extracellular matrix (ECM) [49]. EMT is essential during embryonic development, where these transient genetic changes are closely regulated and associated with several morphogenetic events. Epithelium is the tissue phenotype of early embryos and primitive adults of the chordate phylum. A second tissue type is produced by EMT in higher chordates, such as vertebrates. During gastrulation the primary mesenchymal cells in vertebrates migrate from the primitive streak to differentiate into the mesodermal and endodermal epithelia to set up the three germ layers [49, 50].

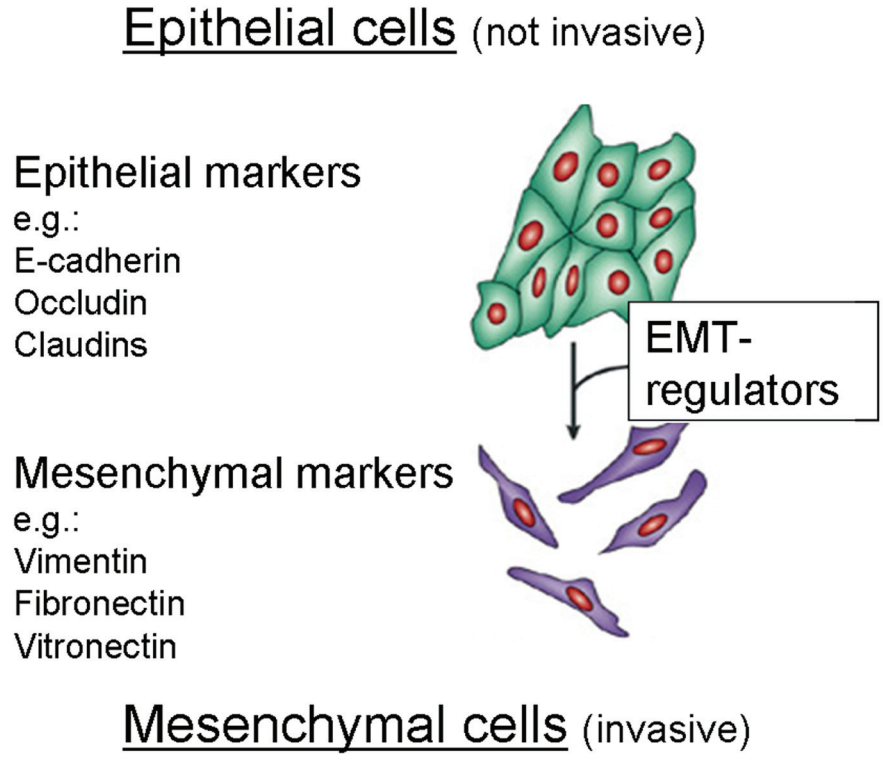

Figure 1-1: During epithelial-mesenchymal-transition epithelial cells loose their epithelial characteristics and gain mesenchymal markers leading to migratory and invasive abilities. Modified from Nieto *et al*. [88].

Besides being essential during early development, it is known that EMT is also an important mechanism during early steps of tumor progression where tumor cells disseminate from the primary tumor [45, 139-141]. Local invasion can be considered an initial and essential step in the malignancy of carcinomas, leading to the generation of usually fatal distant metastases. The process of acquisition of the invasive phenotype by epithelial tumors can be regarded as a pathologic version of the EMT in embryogenesis.

The epithelial tissue forms sheets of cells closely attached to each other by cell-cell adhesions, e.g. adherens junctions and desmosomes, showing an apical-basal polarity. Therefore, a hallmark of EMT, promoting tumor cell migration and invasion, is the loss of this cellular organization prior to migration. One of the key events during EMT is the functional loss of the homophilic cell adhesion molecule E-cadherin, which is known to be a suppressor of invasion during carcinoma progression [14]. In particular, transcriptional repression has recently emerged as a fundamental mechanism for the dynamic silencing of the E-cadherin gene during tumor progression.

1.5 E-CADHERIN

E-cadherin, a 120kDa transmembrane glycoprotein, belongs to the family of classical/type I cadherins [89, 113], with its gene (*CDH1*, GenBank Accession DQ090940) mapped to human chromosome 16q22.1 [13]. Members of this family exhibit calcium-dependent homophilic interactions and are involved in selective cell-cell recognition and adhesion [136]. E-cadherin, a caretaker of the epithelial phenotype, is located in the adherens junctions of epithelia and its function is required for the maintenance of stable cell-cell adhesion and epithelial cell polarity [103, 137].

As mentioned above, loss of E-cadherin is a key process during EMT in embryonic development by which cells loose their epithelial features and become motile, so as to leave the epithelium and move through the extracellular matrix.

Cadherin mediated cell adhesion also plays a critical role in the suppression of tumor invasion, its loss of function coincides with increased tumor malignancy. Down-regulation or inactivation of E-cadherin, which is observed in most epithelial cancers, results in marked phenotypic changes and leads to increased tumor malignancy. There are many examples of carcinomas, e.g. breast, uterine cervix or gastric carcinomas, in which the occurrence of altered E-cadherin expression has been correlated with low histological differentiation, increased risk of local invasion and metastatic disease, recurrence and poor prognosis [40, 55, 61, 62, 85]. Several research groups have shown that regain of functional E-cadherin suppresses invasion in many tumor cell types [52, 54]. Cavallaro and Christofori recently suggested that down-regulation of E-cadherin might even actively participate in the induction of tumor progression by transducing specific signals which, in turn, support tumor invasion [25].

There are multiple mechanisms leading to functional inactivation of E-cadherin during tumor progression, such as gene mutations [12], chromatin rearrangements [27], promoter hypermethylations [135, 152], posttranslational truncation [114] or modification [43], as well as direct transcriptional repression [5, 21, 26, 51].

The human E-cadherin promoter (**Figure 1-2**) has been analyzed in several studies [9, 19, 21, 104, 119] in which different regulatory elements were described in the 5' proximal sequence of the promoter. One of the key elements in the E-cadherin promoter is the E-pal element containing the E-box. The E-pal element acts as a strong repressor of transcription and can even overcome the effects of positive factors acting on the proximal promoter [119]. The zinc finger transcription factor Snail (see below) was shown to bind to the E-pal element in the proximal E-cadherin promoter, thereby leading to repression of E-cadherin transcription [21, 101].

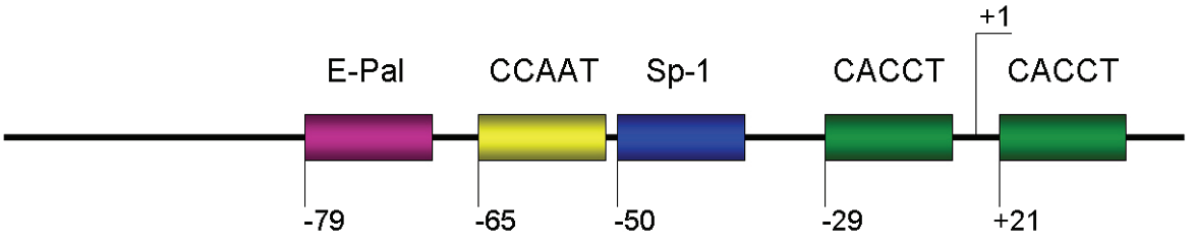

Figure 1-2: The human E-cadherin promoter with putative binding sites for transcription factors and transcription start site (position +1) indicated. Shown are the E-pal element, the CCAAT-box, a Sp-1 consensus sequence, and the SIP1 high-affinity binding sites (two CACCT sequences spaced by 45 nucleotides). The nucleotide sequence of the 5' region of the E-cadherin gene is deposited in the GenBank database (Accession L34545).

1.6 SNAIL

Most prominent EMT-inducing developmental regulators are the Snail-related zinc-finger transcription factors (Snail and Slug) [51, 88]. Snail was first described in *Drosophila melanogaster* as a regulator of mesoderm formation [46], but subsequently Snail homologues were found in other species as well, including mouse and human. It was shown that the Snail family of zinc transcription factors plays a central role in mesoderm formation from flies to mammals and is associated with the appearance of the neural crest [24, 72, 88].

The human Snail gene (*SNAI1*, GenBank Accession AF155233) was isolated and characterized by Twigg and Wilkie in 1999 [143]. The gene spans approximately 6.4kb, contains 3 exons, and has a CpG island upstream of the coding sequence. The Snail gene was mapped, by fluorescence in situ hybridization and radiation hybrid analysis, to human chromosome 20q13.1.

Twigg and Wilkie [143] reported the detection of a single transcript of 1.8kb (GenBank Accession NM_005985) in several human fetal tissues, with the highest expression in kidney, but also in a number of adult tissues, particularly lung and placenta. The Snail open reading frame encodes a protein of 264 amino acids, containing four zinc-finger motifs, with a predicted molecular weight of 29083 Dalton (Swiss-Prot). The zinc-fingers, located in the C-terminal part of the protein, correspond to the C2H2 type (two conserved cysteines and histidines coordinate the zinc ion) and function as specific DNA binding motifs.

Snail represses the transcription of E-cadherin by binding to E-box elements found in the 5' proximal sequence of the E-cadherin promoter, which corresponds to a core of six bases (CANNTG, [5, 21, 82]), thereby triggering a complete EMT with the acquisition of invasive and tumorigenic properties [5, 21, 30, 99]. The repressor activity of Snail not only depends on the zinc-finger motifs, but also on the N-terminal Snail/Gfi-1 (SNAG) domain (**Figure 1-3**). Peinado *et al*. [99] showed that mammalian Snail requires histone deacetylase (HDAC) activity to repress E-cadherin promoter activity. They demonstrated that an interaction between Snail, histone deacetylase 1 (HDAC1) and HDAC2, and the corepressor mSin3A. This interaction was shown to be dependent on the SNAG domain of Snail, indicating that the Snail transcription factor mediates the repression by recruitment of chromatin-modifying activities, forming a multimolecular complex to repress E-cadherin expression [99].

Snail is considered to be a key regulator of EMT by directly down-regulating not only E-cadherin expression but also the epithelial marker genes occludin and claudins [57, 90], which define tight junction components. Hereby the epithelial cells will loose their characteristics, namely cell polarity and cell adhesion. On the molecular level down-regulation of the tight junction components is independent of E-cadherin down-regulation, however, one has to speculate that both components will influence each other. Additionally, transfection of Snail results in down-regulation of other epithelial markers like desmoplakin [21] and MUC-1 as well as cytokeratin-18 [48]. On the other hand, the expression of mesenchymal markers, such as vimentin and fibronectin, is induced [21].

More recently, Snail has been implicated in the progression of human tumors due to its regulatory action promoting the induction of EMT. In humans, Snail mRNA expression has been detected in biopsies or resected tissue samples from patients with breast cancer [15], gastric cancer [120], hepatocellular carcinoma [64], oral squamous cell carcinoma [158], ovarian carcinoma [35], and in melanoma cell lines [110].

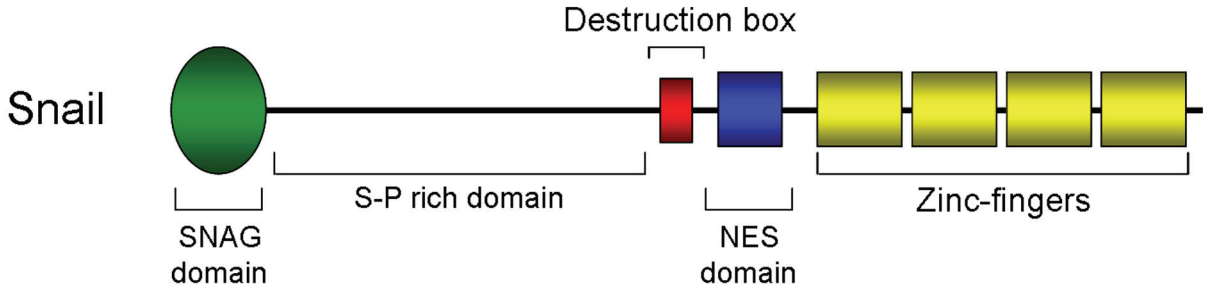

Figure 1-3: Scheme of the main structural domains found in mammalian Snail. Modified from Peinado *et al*. [100]. NES, nuclear export sequence; S-P, serine-proline; SNAG domain, Snail/Gfi-1 domain

1.6.1 Regulation of Snail

Cellular localization and phosphorylation

Control of the nuclear localization of specific proteins is an important mechanism in the regulation of many signal transduction pathways. Transcription factors can not function until they are translocated to the nucleus in response to specific signals

[144]. The translocation of proteins through nuclear pore complexes is mediated by a selective mechanism that is controlled by saturable receptors and specific signals, which are termed nuclear localization signals (NLSs) and nuclear export signals (NESs) [81, 159]. Nuclear localization is essential for Snail's transcriptional function on target genes, e.g. E-cadherin. Yamasaki *et al*. [155] proposed that the zinc-finger domain of Snail, containing all four zinc-fingers, is necessary and sufficient for the nuclear localization of the Snail protein whereas the N-terminal region, containing the SNAG domain, is not. They further demonstrated that Snail binds directly to importin (karyopherin) β in a zinc-finger domain-dependent manner. Ko *et al*. [71] reported two nuclear localization motifs embedded within the Snail sequence. One typical bipartite NLS motif is located at the N-terminal of Snail, where it overlaps with the SNAG domain (residues 8-16), while a basic cluster NLS motif is found proximal to the zinc-finger domains (residues 151-152). Mutational inactivation of these NLS motifs resulted in a decrease of nuclear and total Snail protein levels as well as attenuated Snail repressor activity on an E-cadherin promoter construct. Additionally, Yang *et al*. [157] could show that p21-activated kinase 1 (Pak1) regulates the repressor activity of Snail by phosphorylating on Ser246. Pak1 phosphorylation of Snail supports Snail's accumulation in the nucleus as well as its repressor functions.

Nuclear export of a transcription factor can be regulated, in some cases, by phosphorylation of Ser/Thr residues [60, 92, 162]. Phosphorylation is an ideal mechanism for controlling events during migration and metastasation, as it can be switched on or off almost immediately. Domínguez *et al*. [32] reported that the Snail protein contains a NES (**Figure 1-3**) in its N-terminal half and an adjacent serin-rich domain, which makes the NES sterically accessible after phosphorylation. Zhou *et al*. [163] could show that Snail is phosphorylated by glycogen synthase kinase-(GSK) 3β at two consensus motifs to dually regulate the function of this protein. Phosphorylation of the first motif regulates its β-Trcp mediated ubiquitination, whereas phosphorylation of the second motif controls its subcellular localization (**Figure 1-4**).

Protein stability and functional activity of Snail are therefore tightly regulated by its subcellular localization and its phosphorylation status. Therefore, when studying Snail expression in tissue samples or cell lines, it is necessary to analyze the active, i.e. nuclear localized, Snail protein, as mRNA levels of Snail might not correlate to the active protein.

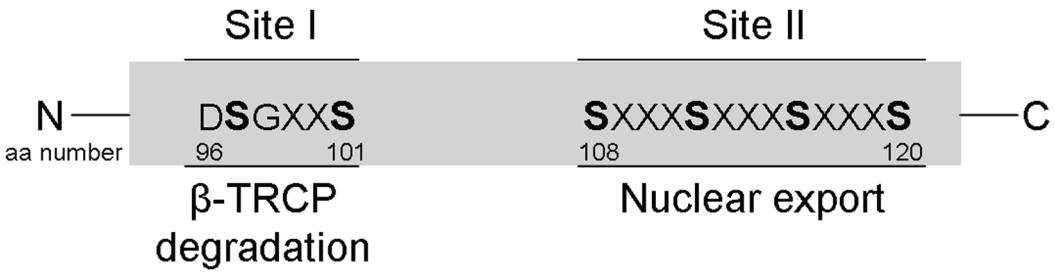

Figure 1-4: GSK-3β phosphorylation consensus sites in human Snail. Site I contains two serine residues that overlap with a destruction box (Figure 1-3) recognized by β-Trcp. Site II contains four serine residues and lies next to a NES. Modified from Schlessinger and Hall [126].aa, amino acid

Estrogen receptor alpha (ERα) signaling and MTA3 expression

The temporal and tissue-specific actions of estrogen are mediated by estrogen receptors (ERs) α and β. The ERs are steroid hormone receptors that modulate the transcription of target genes when bound to ligand [6]. The estrogen-ER dimer complex binds to specific DNA sequences, the estrogen response elements (EREs), which are located in the regulatory regions of estrogen-sensitive genes [98]. The ERs mediate transcriptional regulation of a range of genes, leading to modulation and regulation of multiple physiological processes, such as reproductive organ development and function, bone density. Unfortunately, a range of ER regulated genes are, directly or indirectly, associated with proliferation and invasion in breast and endometrial cancer [98]. At least for breast cancer, ERα rather than ERβ appears to be the predominant regulator of estrogen-induced genes [98].

The metastasis associated protein 3 (MTA3) is a member of a small protein family in mammals. All three members of this family, MTA1, MTA2, and MTA3, have been reported as subunits of MI-2/NuRD [41, 56, 142, 148, 154]. The vertebrate Mi-2/NuRD complex is a multi-subunit protein complex containing both histone deacetylase (HDAC) and nucleosome-dependent ATPase subunits [142, 149, 154, 161]. Current models predict that this complex functions primarily in transcriptional repression after recruitment by site-specific DNA binding proteins.

Fujita *et al*. showed that MTA3 expression is tightly linked to ERα activation and they found that ERα directly binds to and regulates the MTA3 promoter [41, 42]. Additionally, they identified a regulatory pathway that explains how ERα status controls EMT in human breast cancer cell lines, which might contribute to elucidation of how ERα-negative breast carcinomas become metastatic and why they have a generally poor prognosis [74]. They demonstrated that the absence of ERα or of MTA3 leads to aberrant expression of Snail, resulting in loss of E-cadherin expression (**Figure 1-5**) [41]. Repression of Snail is seen after MTA3 or ERα transfection, restoring E-cadherin expression. They found evidence that MTA3 associates with the Snail promoter through unidentified DNA-binding proteins, repressing Snail expression in a HDAC-dependent manner [41]. These results may explain how ERα status controls EMT and invasive growth in human breast cancer and possibly other estrogen-dependent cancers.

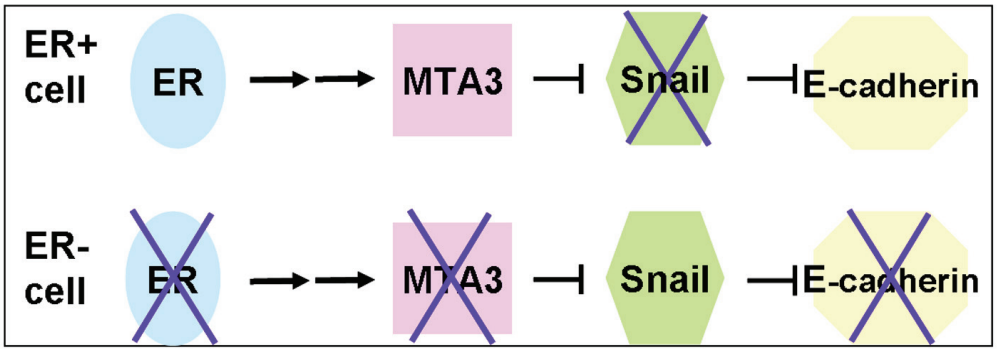

Figure 1-5: Schematic model of the pathway leading from activation of ER to the transcription of E-cadherin. Modified from Fujita *et al*. [41].

Other regulatory pathways

Several other regulatory pathways of Snail expression have been proposed and analyzed during the last years.

One of the main molecules controlling EMT steps is transforming growth factor-β (TGF-β). TGF-β can have both positive and negative effects on tumorigenesis, acting early as a tumor suppressor, but later as a stimulator of tumor invasion [1]. Peinado *et al*. [102] demonstrated that TGF-β1 induces the activity of Snail promoter. They proposed that MAPK and phosphatidylinositol 3-kinase (PI3K) signaling pathways are implicated in TGF-β1-mediated induction of Snail promoter, probably through Ras activation and its downstream effectors.

Also downstream targets of receptor tyrosine kinases (RTK) are supposed to regulate Snail expression. One example is Akt, a downstream target of PI3K, which was shown to be frequently activated in human cancers. Grille *et al*. [47] found evidence that Snail is induced by constitutively active Akt. However, the mechanisms by which Akt activates the Snail promoter remain unclear.

Highly aggressive tumors rapidly outgrow their blood supply, leaving the cells starved of oxygen – a condition known as hypoxia [11]. Hypoxia is known to stabilize hypoxia-inducible factor-1α (HIF-1α) [160], which binds to the hypoxia-response elements of various target genes, inducing gene transcription. Imai *et al*. [59] demonstrated that hypoxia induces down-regulation of E-cadherin, via up-regulation of the transcriptional repressor Snail. However, the molecular mechanism how Snail responds to hypoxia is not yet clear.

1.7 AIM OF THE STUDY / THESIS

The zinc-finger transcriptional repressor Snail is considered as an important regulator of invasiveness during tumor progression due to its inhibitory action on E-cadherin expression. Due to a lack of Snail specific antibodies to reveal protein expression in tumor samples a direct cellular comparison between E-cadherin down-regulation and endogenous nuclear Snail expression at the protein level in cancer tissues has not been possible until now. The Snail specific antibody which was recently established in our lab allows a direct cellular comparison between E-cadherin down-regulation and endogenous nuclear Snail expression at the protein level in sections of formalin-fixed, paraffin-embedded tissues. The aim of this work was to analyze for the first time the active, i.e. nuclear localized, Snail protein in tissue samples of human gynaecological cancers.

In a first study I intended to gain further insight into the role of Snail in the progression of endometrial cancer, the most common gynaecological cancer. I examined the expression of E-cadherin, Snail and estrogen receptor alpha (ERα), which was shown to be involved in the regulation of Snail, in primary tumors and metastases of estrogen-related endometrioid carcinomas, using immunohisto-chemical analysis. In *in vitro* studies on endometrial carcinoma cell lines I focused on the putative regulatory pathway of Snail expression via ERα signaling and MTA3 expression.

In a second immunohistochemical study I wanted to elucidate the role of Snail in E-cadherin down-regulation and cancer progression in ovarian carcinomas, the leading cause of death among gynaecological malignancies.

In a third study I analyzed Snail expression in a natural model for migration and invasion, human placental development. Not much is known so far about the expression pattern of Snail or its role in regulating E-cadherin expression in invading extravillous trophoblast cells. Aim of this subproject was to investigate the expression pattern of E-cadherin and Snail in placental tissue derived from pregnancies complicated by preeclampsia or HELLP syndrome compared to placentas from normal pregnancy courses.

2 MATERIALS AND METHODS

2.1 BUFFERS AND SOLUTIONS

The following list specifies common buffers used consistently throughout the manuscript. For special buffers, refer to the end of the corresponding section. Addresses of providers of the material used are listed in section **2.12**.

Citrate Buffer
4.2g Citric Acid Monohydrate (#1.00244.1000, Merck KGaA)
in 2l aqua dest.
pH = 6.0 (NaOH)

PBS
137mM NaCl (#1.06404.5000, Merck KGaA)
8.2mM Na_2HPO_4 x $2H_2O$ (#1.06580.1000, Merck KGaA)
1.8mM KH_2PO_4 (#1.04873.1000, Merck KGaA)
pH=7.4
A 20x solution was prepared, autoclaved and stored at room temperature.

SDS-polyacrylamide gel

Separating gel (10%, 10ml)
4.88ml aqua dest.
2.5ml 1M Tris (#75825, UBS Corporation) pH 8.8
2.5ml 40% Acrylamide/Bis solution 37.5:1 (#161-0148, BioRad Lab.)
50µl 20% SDS (#A0675,1000, AppliChem)
60µl 10% APS (#A7460, Sigma-Aldrich)
6µl TEMED (#161-0801, BioRad)

Assemble gel (5ml)
3.05ml aqua dest.
1.25ml 0.5M Tris (#75825, UBS Corporation) ph 6.8
0.63ml 40% Acrylamide/Bis Solution 37.5:1 (#161-0148, BioRad Lab.)
25µl 20% SDS (#A0675,1000, AppliChem)
40µl 10% APS (#A7460, Sigma-Aldrich)
4µl TEMED (#161-0801, BioRad)

Laemmli loading buffer
60mM Tris (#75825, UBS Corporation) pH=6.8
8.7% v/v glycerol (#1.04092.1000, Merck KGaA)
0.72M 2-mercaptoethanol (#M7154, Sigma-Aldrich)
80µg/ml bromphenol blue (#B5525, Sigma-Aldrich)
2% w/v SDS (#A0675,1000, AppliChem)
The buffer was prepared 5x concentrated and stored in aliquots at 4°C.

SDS running buffer
193mM glycine (#1.04201.1000, Merck KGaA)
25mM Tris (#75825, UBS Corporation)
0.05% w/v SDS (#A0675,1000, AppliChem)
A 10x buffer was prepared and stored at room temperature.

Blot-buffer
192mM glycine (#1.04201.1000, Merck KGaA)
25mM Tris (#75825, UBS Corporation)
0.2% w/v SDS (#A0675,1000, AppliChem)
20% v/v methanol (#1.06009.1000, Merck KGaA)
The buffer was stored at 4°C.

Stripping buffer
10% v/v methanol (#1.06009.1000, Merck KGaA)
10% v/v acetic acid (glacial) (#1.00063.1000, Merck KGaA)
in aqua dest.
The buffer was stored at room temperature.

TBS
20mM Tris (#75825, UBS Corporation)
137mM NaCl (#1.06404.5000, Merck KGaA)
pH=7.3
A 10x buffer was prepared, autoclaved, and stored at room temperature.

TBST
TBS supplemented with 0.1% v/v Tween-20 (#8.22184.1000, Merck KGaA).
The buffer was stored at room temperature.

TBST-M
TBST containing 2.5-5% w/v non-fat dried milk (#60219, Töpfer)
The buffer was prepared freshly before use and stored at 4°C.

2.2 TISSUE SAMPLES

2.2.1 Endometrial cancer

113 formalin-fixed, paraffin-embedded tissue specimens, consisting of 87 primary endometrial adenocarcinomas (endometrioid subtype, WHO) and 26 unrelated metastases, diagnosed between 1991 and 2003 were selected from the files of the Institute of Pathology, Technical University of Munich, Germany. Some cases were obtained during intrauterine curetting. Metastases were located in the retroperitoneal area (n=13), distant lymph nodes (n=4), lungs (n=3), central nervous system (n=3), peritoneal cavity (n=2), or other locations (n=1). Except two cases (clear cell type), all metastases were classified as endometrioid subtype. Grading and staging of the primary carcinomas were ascertained according to the proposals of the WHO and the UICC [134, 138].
Formalin-fixed, paraffin-embedded specimens of the primary endometrial tumors and the metastases were immunohistochemically analyzed for expression of E-cadherin,

Snail, and ERα. Additionally, 11 primary tumors, 5 ERα positive and 6 ERα negative cases according to the IHC results, were analyzed for expression of E-cadherin, Snail, MTA3 and ERα using a recently established method [8] of protein extraction from formalin-fixed, paraffin-embedded tissue specimens following Western Blot analyses.

2.2.2 Ovarian cancer

51 patients who had undergone primary surgery for newly diagnosed advanced stage (FIGO IIIC and IV) ovarian cancer between 1998 and 2001 at the University Hospital of the Technical University of Munich, Germany were eligible for this retrospective analysis. Exclusion criteria were a second malignancy, or chemotherapy or radiotherapy within the last 6 months prior to surgery. Follow-up data was available for all patients, with a median follow-up time of 55 months. In 47 cases, specimens of the primary tumor as well as the corresponding metastases were available, in 1 case only the primary tumor was present, and in 3 cases only specimens of metastases were available. Metastases were located in the peritoneum (n=17), omentum (n=25), distant lymph nodes (n=6) and uterus (n=2).
Formalin-fixed, paraffin-embedded specimens of the primary ovarian tumors and corresponding metastases were immunohistochemically analyzed for expression of E-cadherin and Snail.

2.2.3 Placenta

Placental tissues were obtained from 19 women who underwent delivery at the 1st Department of Obstetrics and Gynaecology of the LMU Munich, Germany. Specimens were collected immediately after delivery from five patients with HELLP syndrome (mean date of delivery: 33.8±2.9 weeks of gestation), seven patients with preeclampsia (mean date of delivery: 33±3.2 weeks of gestation) and seven patients following a normal course of pregnancy (mean date of delivery: 38.2±3 weeks of gestation). The study had the approval of the local ethical committee of the LMU Munich, Germany (No. 158/00) and informed consents from the patients were obtained.
Formalin-fixed, paraffin-embedded specimens of the placental tissues were immunohistochemically analyzed for expression of E-cadherin and Snail.

2.3 IMMUNOHISTOCHEMISTRY

4µm sections of formalin-fixed and paraffin-embedded material were analyzed. Deparaffination of the sections was done using xylene and graded alcohol series. After antigen retrieval by boiling in citrate buffer in a pressure cooker for 4 minutes, sections were incubated in either 1% hydrogen peroxide (for E-cadherin) for 15 minutes to block endogenous peroxidase or in 3% normal goat serum (#S-1000, Vector) (for Snail) for 20 minutes to reduce non-specific background staining. In case of 1% hydrogen peroxide blocking, sections were washed afterwards 3 x 2 minutes in PBS. For detection of specific immunoreactivity, the specimens were incubated with either a monoclonal anti-E-cadherin antibody (mouse, 1:1500) (#610182, BD Biosciences Pharmigen) at room temperature for 1 hour or a monoclonal anti-Snail antibody (rat, 1:20) (hybridoma supernatant Sn9H2, Dr. Kremmer, GSF, Munich

[121]) at room temperature for 2 hours. Antibodies were diluted in freshly prepared 1%BSA/PBS. Afterwards, slides were washed 3 x 2 minutes in PBS. Both antibodies were detected using the avidin-biotin-complex (ABC) peroxidase method (#PK-6102 (anti-mouse), #PK-6104 (anti-rat) ABC Elite Kit, Vector). Final staining was developed with the Sigma FAST DAB peroxidase substrate kit (#D-4293, Sigma-Aldrich). Nuclei were counterstained with haematoxylin for 10 seconds and rinsed briefly with tap water. Finally, tissue sections were dehydrated through graded alcohol series, mounted with pertex balm and covered with glass slips.
ERα immunohistochemistry was performed using the automated slide preparation system Benchmark® XT from Ventana Medical Systems. The monoclonal anti-ERα antibody (#EI629C01, DCS Innovative Diagnostik-Systeme) was used at a 1:20 dilution. Detection of the primary antibody and development of final staining was done using the Enhanced V-Red (Alk. Phos. Red) Detection Kit (#760-031) from Ventana Medical Systems for automated slide preparation.

Albumin from bovine serum (BSA) (#A3912-100G, Sigma-Aldrich)

2.3.1 Immunohistochemical evaluation

Tumor tissue (endometrial and ovarian cancer):

E-cadherin immunoreactivity was evaluated as following:
Expression of E-cadherin was assessed using a semiquantitative scoring system, ranging from 0, 1+, 2+, 3+. Only membranous staining was evaluated.
E-cadherin expression was scored as follows:

- 0, no immunoreactivity, or immunoreactivity of <10% of tumor cells
- 1+, low intensity immunoreactivity of ≥ 10% of tumor cells
- 2+, medium intensity immunoreactivity of ≥ 10% of tumor cells
- 3+, high intensity immunoreactivity of ≥ 10% of tumor cells

All cases were summarized in two groups, showing preserved E-cadherin expression (Score 3+) or reduced E-cadherin expression (Scores 0, 1+, 2+).

Snail immunoreactivity was evaluated as following:
For Snail immunoreactions, staining was graded as negative (no immunoreactivity, or immunoreactivity of <1% of tumor cells), or positive (immunoreactivity of ≥1% of tumor cells). Snail staining was graded as positive only when nuclear staining was detectable.

ERα immunoreactivity was evaluated as following:
Intensity and distribution pattern of the specific immunostaining was evaluated using a semi-quantitative immunoreactivity score (IRS) (score 0-12), which was described by Remmele and Stegner in 1987 and was used in previous investigations on estrogen alpha and beta receptor expression in the normal human endometrium [118]. The IRS score was calculated by multiplying staining intensity (SI) and percentage of positivity (PP); in detail SI is graded as 0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). Counting approximately 200 cells the PP is graded as 0 = no stained cells, 1 = less or equal 10% of stained cells, 2 = between 11 and 50% of cells are stained, 3 = 51 – 80% of cells are stained, 4 = more than 80% of cells are stained.
Score values from 4-12 were considered as ERα positive, 0-3 as ERα negative.

Placental tissue:

Snail and E-cadherin immunoreactivity was evaluated as following:

To monitor the differences of E-cadherin and Snail immunoreactivity in normal placental tissue compared to pathologic placental tissue, the semi-quantitative immunoreactivity score (IRS) (score 0-12) was used for evaluating Snail and E-cadherin immunoreactivity in this tissue type. As described above, the IRS score was calculated by multiplying staining intensity (SI) and percentage of positivity (PP). For E-cadherin only membranous staining and for Snail only nuclear staining was evaluated.

2.3.2 Statistical analysis

For statistical evaluation the primary tumors of endometrial cancer were divided into two groups with either 1-5% Snail positive cells or >5% Snail positive cells. This classification was done because nearly half of the cases was found with very high percentages of Snail positive carcinoma cells while the rest displayed only low percentages of Snail positive carcinoma cells. These discrepancies might be explained by the fact that EMT is a local event and therefore, Snail is often only locally expressed.
For metastases of endometrial cancer as well as for primary tumors and corresponding metastases of ovarian cancer these discrepancies were not observed. Therefore, they were classified as either Snail-positive or Snail-negative for statistical evaluation.

Continuous variables are reported as median with range and categorical data are expressed as frequencies and percentages. Chi-Square test and if appropriate Fisher Exact test were applied to test for bivariate associations of categorical parameters. Analysis of survival times was performed using the method of Kaplan-Meier and comparison of survival between patient subgroups was done by Log-Rank test and Cox-proportional hazard regression. Hazard ratios and median survival were reported with 95% confidence intervals. All tests were performed two-tailed at a 5% level of significance. Analyses were conducted using SPSS version 14.0 (SPSS Inc.).

2.4 PROTEIN EXTRACTION FROM FFPE TISSUE

Full length proteins from 10µm sections of formalin-fixed, paraffin-embedded tissues were extracted as recently described [8].
Tumor cell areas (more than 80% tumor cells) were selected according to ERα immunoreactivity (positive or negative) and marked on the ERα stained reference slide. 10µm FFPE sections were cut of the chosen cases and deparaffination of the sections was done using xylene and graded alcohol series. The unstained sections were rehydrated in aqua dest. for 10 minutes. The area of interest was scratched from the wet, unstained slide (according to the marked area on the ERα stained reference slide) with a needle and transferred to a 1.5ml tube containing appropriate amounts of Qproteome FFPE tissue kit (#37623, Qiagen) buffer. The samples were incubated on ice for 5 minutes and mixed by vortexing carefully. Thereafter, the samples were transferred to a 100°C hot waterbath and incubated for 20 minutes. Afterwards, the tubes were incubated at 80°C for 2 hours in a thermomixer with

agitation at 750rpm. The samples were then transferred on ice and centrifuged at 4°C and 14000 x g for 15 minutes. The supernatant, containing the extracted proteins, was transferred to a fresh 1.5ml tube and stored at –20°C. Western Blots were performed as described below (refer to section **2.11**).

2.5 IMMUNOFLUORESCENCE DOUBLE STAINING

For the identification of E-Cadherin and Snail expressing cells in placental tissue, cryosections of placenta were examined by immunofluorescence. All samples were fixed in 5% buffered formalin. Antibodies and antibody dilution used in the experiment are listed in **Table 2-1**. All antibodies were diluted in Antibody Diluent with Background Reducing Components (#S3022, DakoCytomation). Primary antibodies were incubated with the slides overnight at 4°C. After washing with PBS three times for 2 minutes, Cy3-labeled goat anti-mouse IgG or Cy2-labeled goat anti-rat IgG served as secondary antibody. Alternatively, Cy2-labeled goat anti-mouse IgG was used as secondary antibody in some cases. After secondary antibody incubation slides were washed again with PBS three times for 2 minutes. The slides were finally embedded in mounting buffer containing 4',6-Diamino-2-phenylindole (DAPI) (#H1200, Vector) resulting in staining of the nuclei. Slides were examined with a Zeiss Axiophot photomicroscope. Digital images were obtained with a digital-camera system (Axiocam, Zeiss) and saved on computer.

Table 2-1: Antibodies and antibody dilutions used for immunofluorescence double staining.

Primary Antibody	Species	Dilution
Snail (Dr. Kremmer, GSF, Munich [121])	Rat	1:60
E-Cadherin (#205601, Calbiochem)	Mouse	1:800
Cytokeratin 7 (#CK7-OVTL-CE, Novocastra)	Mouse	1:30
Cytokeratin-Cy3-labeled (Clone A45-B/B3, Micromet)	Mouse	1:1000
Secondary Antibody		
anti-mouse IgG-Cy3-labeled (#115-167-003, Dianova)	Goat	1:200
anti-mouse IgG-Cy2-labeled (#115-227-003, Dianova)	Goat	1:200
anti-rat IgG-Cy2-labeled (#112-227-003, Dianova)	Goat	1:200

2.6 CELL CULTURE

2.6.1 Cell culture conditions

Cells were grown at 37°C in an atmosphere of 5% CO_2 using a Heraeus CO_2 incubator (type BBK 6220, Kendro Laboratory Products).

2.6.2 Cell lines and growth media

The following cell lines were used: Ishikawa (Human Asian endometrial adenocarcinoma, European Collection of Cell Cultures (ECACC) No. 99040201), Ishikawa ER- (Human endometrial adenocarcinoma, ECACC No. 98032302), HEC-1-A (Human endometrial adenocarcinoma, American Type Culture Collection (ATCC) No. HTB-112) and HEC-1-B (Human endometrial adenocarcinoma, ATCC No. HTB-113).
All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, #31966-021, Gibco), 10% fetal calf serum (FCS, #3302-P260317, PAN Biotech) and 2.5 ml penicillin/streptomycin (10,000 units/ml penicillin G sodium/10,000 µg/ml streptomycin sulphate; #15140-122, Gibco).

2.6.3 Passaging of cells

Cells were washed with 10ml prewarmed PBS (37°C), trypsinized for approximately 5 minutes (1ml trypsin per 25cm^2 tissue culture flask, 37°C), harvested in prewarmed culture medium (37°C), splitted in an appropriate ratio and seeded in 25cm^2 tissue culture flasks.

Dulbecco's PBS (#H15-002, PAA Laboratories)

Trypsin-EDTA (#L11-004, PAA Laboratories)

2.6.4 Preparation of frozen stocks

Cells grown to 80% confluency in a 75cm^2 tissue culture flask were washed with PBS, trypsinized, harvested in 10ml culture medium (4°C) and transferred to a 15ml Falcon tube (on ice). After centrifugation at 258 x g and 4°C for 3 minutes they were resuspended in 5ml freezing medium and aliquoted into 1ml freezing vials. The vials were placed at –20°C for 4 hours and then transferred to –80°C. On the following day the vials were placed in liquid nitrogen for long time storage.

Freezing medium
90% v/v FCS (#3302-P260317, PAN Biotech)
10% v/v DMSO (#20385, SERVA)

2.6.5 Starting cultures from frozen stocks

15ml Falcon tubes containing 9ml culture medium (37°C) were prepared before thawing. Vials with frozen cells were transferred to a 37°C water bath. After thawing, cells were transferred to the medium containing tubes, centrifuged at 258 x g for 3 minutes, resuspended in fresh medium, and seeded in 25cm^2 tissue culture flasks. The medium was changed the following day.

2.7 WOUND-HEALING ASSAY

Wound-healing assays were used to determine cell migration.

2.7.1 Ishikawa and Ishikawa ER- cells

1 x 10^6 Ishikawa or Ishikawa ER- cells per well of a 6-well plate were seeded in 2ml culture medium and were grown to confluence. Triplicate wells of Ishikawa or Ishikawa ER- cells were grown 24 hours without serum before wounding to minimize cell proliferation. A scrape was made in the cell layer with a 200µl pipette tube followed by three washing steps with serum free medium. The cells were kept in the absence of serum for 48 hours followed by microscopy (Axiovert 135 microscope, Zeiss, Jena, Germany) to monitor the advance of the cells into the wound. Pictures of the same site of the wound were taken directly (0 hours), 24 hours, and 48 hours after scraping. Digital images were obtained with a digital-camera system (Axiocam MRc5, Zeiss, Jena, Germany) and saved on computer. The two borders of the wound were marked by lines on the digital images. Quantification was done by counting the cells that had migrated into the wound.

2.7.2 Ishikawa cells untreated and treated with fulvestrant

Ishikawa cells were cultured in penicillin/streptomycin-free DMEM medium containing 10% FCS and treated with either 1µM fulvestrant (#1047, Tocris Bioscience) (solved in $EtOH_{absolute}$) or, as a control, with an equal volume of $EtOH_{absolute}$ (#1.00983.1000, Merck KGaA). After two days of treatment, 1 x 10^6 Ishikawa cells per well of a 6-well plate were seeded in 2ml of penicillin/streptomycin-free DMEM medium containing 10% FCS and either 1µM fulvestrant or an equal volume of $EtOH_{absolute}$. The cells were grown to confluence for approximately 24 hours. On day three of fulvestrant treatment, the medium was aspirated from the cells and replaced by 2ml penicillin/streptomycin- and serum free DMEM containing either 1µM fulvestrant or an equal volume of $EtOH_{absolute}$. Triplicate wells of fulvestrant-treated and control Ishikawa cells were grown 24 hours under these serum-free conditions before wounding to minimize cell proliferation. On day four of fulvestrant treatment, a scrape was made in the cell layer with a 200µl pipette tube followed by three washing steps with serum free medium. The cells were kept in the absence of serum, but with either 1µM fulvestrant or with an equal volume of $EtOH_{absolute}$, for 48 hours followed by microscopy (Axiovert 135 microscope, Zeiss, Jena, Germany) to monitor the advance of the cells into the wound. Pictures of the same site of the wound were taken directly (0 hours), 24 hours, and 48 hours after scraping. Digital images were obtained with a digital-camera system (Axiocam MRc5, Zeiss, Jena, Germany) and saved on

computer. The two borders of the wound were marked by lines on the digital images. Quantification was done by counting the cells that had migrated into the wound.

2.8 siRNA ASSAY

2.8.1 Transfection of short interfering RNA (siRNA) using Lipofectamine 2000

The day before transfection 4 x 10^4 Ishikawa cells per well of a 12-well plate were seeded in 1ml of penicillin/streptomycin-free DMEM medium containing 10% FCS. Cells were grown to 50% confluency for approximately 24 hours.
For preparation of solution A 40pmol of siRNA oligomer were diluted in 50µl Opti-MEM (#51985, Gibco) for each transfection and mixed gently.
The Lipofectamine 2000 reagent (#11668-019, Invitrogen) was mixed gently before use. For preparation of solution B 3µl of Lipofectamine 2000 reagent were diluted in 50µl Opti-MEM for each transfection and mixed gently.
Solution A and B were combined after an incubation time of 5 minutes at room temperature and mixed gently. An incubation step of 20 minutes at room temperature allows the formation of RNA-liposome complexes. The RNA-Lipofectamine 2000 complexes were then added to each well containing cells and medium and mixed gently by rocking the plate back and forth.
The cells were incubated with the complexes for 16 hours at 37°C, 5% CO_2. Following the incubation, the medium was replaced by normal growth medium without antibiotics.

Cells were harvested on day 3 to day 7 after transfection. They were lysed in T-PER buffer (refer to section **2.11.1**) and analyzed by Western Blot (**2.11.**).

As an ERα-specific siRNA a commercially available pre-designed siRNA from Ambion (#AM16708) was used, targeting exons 1, 2 of human ERα.

As a control siRNA a commercially available non-targeting siRNA from Dharmacon (#D-001220-01-05) was used.

2.9 FULVESTRANT ASSAY

7 x 10^4 Ishikawa cells per well of a 6-well-plate were seeded in 2ml penicillin/ streptomycin-free DMEM medium containing 10% FCS.
After 24 hours fulvestrant (#1047, Tocris Bioscience) (solved in $EtOH_{absolute}$) was added to a final concentration of 1µM. Control cells were treated with an equal volume of $EtOH_{absolute}$ (#1.00983.1000, Merck KGaA).
Treatment of cells was performed for six days. Cells were harvested at approximately 80% confluency on day 3 to day 6 after the first application of fulvestrant and $EtOH_{absolute}$, respectively. They were lysed in T-PER buffer (refer to section **2.11.1**) and analyzed by Western Blot (**2.11.**).
To avoid high cell densities, cells were passaged, whenever necessary, in an appropriate ratio into penicillin/streptomycin-free DMEM medium containing 10% FCS and either 1µM fulvestrant or $EtOH_{absolute}$.

2.10 17β-ESTRADIOL ASSAY

5 x 10^4 Ishikawa cells per well of a 6-well-plate were seeded in 2ml penicillin/ streptomycin-free DMEM medium containing 10% FCS.
After 24 hours 17β-estradiol (#E4389, Sigma-Aldrich) (solved in aqua dest.) was added to a final concentration of 1µM. Control cells were treated with an equal volume of aqua dest..
Treatment of cells was performed for eight days. Cells were harvested at approximately 80% confluency on day 4 to day 8 after the first application of 17β-estradiol and aqua dest., respectively. They were lysed in T-PER buffer (refer to section **2.11.1**) and analyzed by Western Blot (**2.11.**).
To avoid high cell densities, cells were passaged, whenever necessary, in an appropriate ratio into penicillin/streptomycin-free DMEM medium containing 10% FCS and either 1µM 17β-estradiol or aqua dest..

2.11 WESTERN BLOT

2.11.1 Cell lysis

The cell monolayer was washed with 10ml prechilled PBS (4°C). After washing, ice-cold T-PER buffer was added (200µl/confluent 6-well tissue culture plate, 100µl/confluent 12-well tissue culture plate), cells were scraped of and transferred into a 1.5ml tube. Cell lysates were incubated on ice for 10 minutes and centrifuged at 4°C and 13000 x g for 15 minutes. The supernatant fluid, which is the total cell lysate, was transferred to a fresh tube and stored at –80°C.

T-PER Tissue Protein Extraction Reagent (#78510, PIERCE)
Protease inhibitor cocktail was added before use: 1 tablet Complete Mini (#1836153, Roche)/10ml T-PER buffer.

2.11.2 Protein quantitation by the method of Bradford

Protein Assay Dye Reagent Concentrate (#500-0006, BioRad) was diluted 1:5 in aqua dest.. Protein Standard II (1.48 mg BSA/ml, #500-0007, BioRad) was diluted 1:10 in aqua dest.. Samples for the standard curve were prepared as shown in **Table 2-2** and transferred into half microcuvettes. 995µl of the diluted Protein Assay Dye Reagent Concentrate were aliquoted in half microcuvettes and 5µl of unknown test samples were added (dilution 1:200). After vortexing, all samples were incubated at room temperature for 1 minute. The absorbance was measured at 595nm vs. water reference, a standard curve was established, and the sample protein concentration was calculated.

Table 2-2: Sample preparation for protein standard curve.

Sample #	Protein Assay Dye Reagent Concentrate (1:5 dilution in aqua dest.)	Protein Standard II [1.48mg BSA/ml] (1:10 dilution in aqua dest.)	Protein [µg]
1	990µl	10µl	1.48
2	970µl	30µl	4.44
3	950µl	50µl	7.4
4	930µl	70µl	10.36

2.11.3 SDS Gel electrophoresis

Samples were supplemented with Laemmli buffer and separated on a suitable SDS-polyacrylamide gel in SDS running buffer.

2.11.4 Protein transfer from SDS gels to a PVDF-membrane (Western Blotting)

The PVDF-membrane (#162-0184, BioRad) was preincubated in methanol (#1.06009.1000, Merck KGaA) for 15 minutes and equilibrated in blot-buffer for 5 minutes at room temperature. Fiber pads and gel blotting papers (#10426693, Schleicher&Schuell Bioscience) were soaked in blot-buffer. The blot sandwich was prepared as follows: A fiber pad was laid on the black part of the blotting cassette. The SDS gel was carefully detached from the glass plate of the gel electrophoresis unit by a dry gel blotting paper, put on the fiber pad and covered with the PVDF-membrane. Air bubbles were removed by rolling a Falcon tube over the membrane. The membrane was then covered with another gel blotting paper and a fiber pad. Together with a cooling unit the cassette was placed in the buffer tank, completely covered with ice-cold blot-buffer and placed on top of a magnet stirrer. The transfer was carried out with a constant voltage of 100V for 60 minutes. Following the transfer, the membrane was subjected to the blocking procedure.

2.11.5 Immunological detection of immobilized proteins

The non-specific sites on the membrane were blocked in blocking solution (**Table 2-3**) for 2 hours at 4°C. The membrane was incubated with primary antibody (**Table 2-3**) diluted in blocking solution for 16 hours at 4°C on a shaker. After incubation the membrane was washed 3 x 10 minutes with TBST. Incubation with secondary antibody (**Table 2-3**) was performed for 2 hours at 4°C in blocking solution. Afterwards the membrane was washed again 3 x 10 minutes in TBST. Detection of horseradish peroxidase (HRP) activity was performed using the ECL-Plus Western Blotting Detection System (#RPN2132, GE Healthcare) according to the manufacturers instructions. A film (#RPN 3103K, Amersham Biosciences) was

exposed to the membrane in a film cassette for an appropriate period of time, developed and fixed in red light.

Table 2-3: Antibodies, antibody dilutions and blocking conditions used for Western Blot.

Primary Antibody	Species	Dilution	Blocking/ Diluent
Snail (Dr. E. Kremmer, GSF, Munich [121])	Rat	1:50	0.5% BSA/ 2.5% TBST-M
E-Cadherin (#610182 BD Biosciences Pharmigen)	Mouse	1:10000	5% TBST-M
ERα (#E-0646, Sigma-Aldrich)	Rabbit	1:3000	5% TBST-M
MTA3 (#A300-160A, Bethyl Lab.)	Rabbit	1:3000	5% TBST-M
β-actin (#A 5441, Sigma-Aldrich)	Mouse	1:10000	5% TBST-M
Secondary Antibody			
anti-mouse IgG HRP-conjugated (#NA931-1ML, GE Healthcare)	Sheep	1:10000	5% TBST-M
anti-rabbit IgG HRP-conjugated (#7074, Cell Signaling Technologies)	Goat	1:5000	5% TBST-M
anti-rat IgG HRP-conjugated (#NA9350, GE Healthcare)	Goat	1:5000	0.5% BSA/ 2.5% TBST-M

2.11.6 Stripping

After washing the membrane 3 x 10 minutes in TBST it was incubated 3 x 10 minutes in Stripping buffer at room temperature. Afterwards it was washed again 3 x 10 minutes in TBST. The stripped membrane was stored at 4°C or subjected to immunological detection of immobilized proteins (as described in **2.11.5**).

2.12 LIST OF PROVIDERS

Ambion, Austin, TX, USA

Amersham Biosciences, Buckinghamshire, UK

AppliChem, Darmstadt, Germany

BD Biosciences Pharmigen, San Diego, CA, USA

Bethyl Laboratories, Montgomery, TX, USA

BioRad Laboratories, Hercules, CA, USA

Calbiochem, San Diego, CA, USA

Cell Signaling Technologies, Danvers, MA, USA

DakoCytomation, Glostrup, Denmark

DCS Innovative Diagnostik-Systeme, Hamburg, Germany
Dharmacon, Lafayette, CO, USA
Dianova, Hamburg, Germany
GE Healthcare, Buckinghamshire, UK
Gibco, Paisley, Scotland/UK
Invitrogen, Karlsruhe, Germany
Kendro Laboratory Products GmbH, Langenselbold, Germany
Merck KGaA, Darmstadt, Germany
Micromet, Munich, Germany
Novocastra, Newcastle Upon Tyne, UK
PAA Laboratories, Pasching, Austria
PAN Biotech, Aidenbach, Germany
PIERCE, Rockford, IL, USA
Qiagen, Hilden, Germany
Roche Diagnostics, Mannheim, Germany
Schleicher&Schuell Bioscience, Dassel, Germany
SERVA Electrophoresis GmbH, Heidelberg, Germany
Sigma-Aldrich, Munich, Germany
SPSS Inc., Chicago, IL, USA
Tocris Bioscience, Bristol, UK
Töpfer GmbH, Dietmannsried, Germany
USB Corporation, Cleveland, OH, USA
Vector Laboratories, Burlingame, CA, USA
Ventana Medical Systems, Tucson, AZ, USA
Zeiss, Jena, Germany

3 RESULTS

3.1 E-CADHERIN AND ITS REPRESSOR SNAIL IN ENDOMETRIAL CANCER

A total of 87 specimens of primary tumors and 26 specimens of independent metastases of endometrioid adenocarcinomas (EEC) were examined for E-cadherin, Snail and ERα immunoreactivity (**Figure 3-1**). A summary of the histopathological and immunohistochemical features is given in **Table 3-1**.

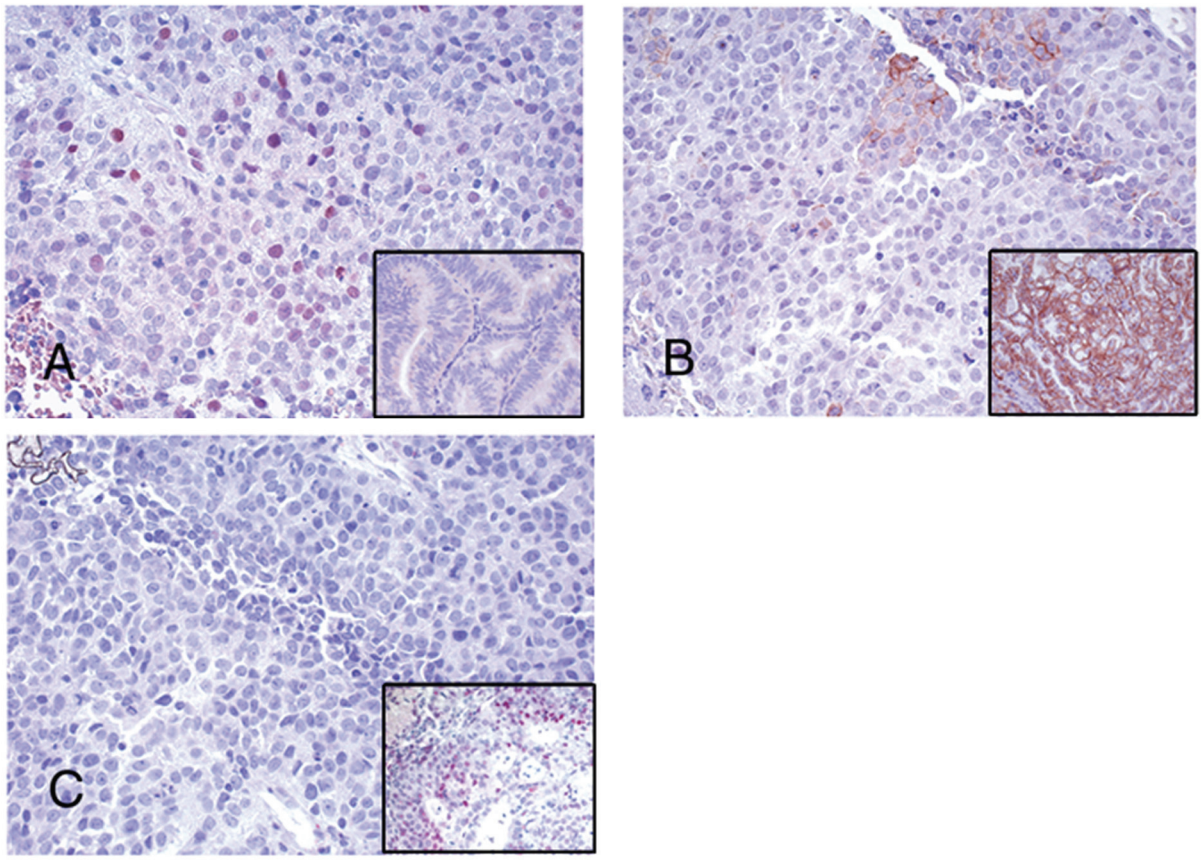

Figure 3-1: (A-C) Consecutive immunostainings of a poorly differentiated endometrial adenocarcinoma (case no. 45):
A: The tumor cells show nuclear immunoreactivity for Snail without cytoplasmatic staining. Note that the nuclear positivity is scattered throughout the tumor. Inset: For comparison, a negative Snail immunostaining is shown in another, higher differentiated tumor (case no. 28) (immunoperoxidase staining, x400). B: Immunostainings with antibodies against E-cadherin show a mostly negative staining. Inset: Note that E-cadherin expression is preserved in better differentiated parts of the same tumor (case no. 45) (x400). C: Immunostainings with antibodies against ERα show a complete negative reaction. Inset: For comparison, a positive ERα staining is shown in a different, well differentiated case (case no. 76) (x400).

Table 3-1. Summary of histopathological and immunohistochemical features in primary tumors and metastases of EECs.
[#]Staging and grading according to UICC classification [134, 138]; [†]Tx represents cases of diagnostic curetting, lost in follow up

	Primary tumors (n=87) n(%)	Metastases (n=26) n(%)
Stage[#]		
I	55(63.2%)	-
II	12(13.8%)	-
III	11(12.6%)	-
IV	1(1.1%)	-
Tx[†]	8(9.3%)	-
Grade[#]		
1	22(25.3%)	6(23.1%)
2	48(55.2%)	12(46.2%)
3	17(19.5%)	8(30.7%)
E-cadherin immunoreactivity		
Preserved (score 3+)	48(55.2%)	9(34.6%)
Reduced (score 0, 1+, 2+)	39(44.8%)	17(65.4%)
Snail immunoreactivity		
Positive	25(28.7%)	14(53.8%)
Negative	62(71.3%)	12(46.2%)
ERα immunoreactivity		
Preserved (score 4-12)	38(43.7%)	15(57.7%)
Reduced (score 0-3)	49(56.3%)	11(42.3%)

3.1.1 E-cadherin immunoreactivity in primary and metastatic endometrial cancer

E-cadherin immunoreactivity was preserved (score 3+) in 55.2% of primary endometrial cancers and in 34.6% of the metastatic lesions. 44.8% of primary tumors showed a reduced E-cadherin immunoreactivity (score 0, 1+, 2+) (**Table 3-1**) but no association was seen between reduced E-cadherin immunoreactivity and tumor stage or tumor grade. Nevertheless, a statistically significant correlation was seen to reduced ERα immunoreactivity in primary tumors of EECs (p=0.01) (**Table 3-2**).
The frequency of cases with abnormal E-cadherin immunoreactivity was higher in the metastatic lesions of EECs. 65.4% of the metastases showed a reduced E-cadherin

immunoreactivity with a statistically significant correlation to a higher tumor grade (p=0.003). In contrast to the primary tumors no association was seen between reduced E-cadherin immunoreactivity and reduced ERα immunoreactivity in metastatic lesions of EECs (**Table 3-1** and **Table 3-2**).

Table 3-2: Statistical correlation between E-cadherin immunoreactivity and histopathological features in primary tumors and metastases of EECs.
***statistically significant (p<0.05); #Staging and grading according to UICC classification [134, 138]; †Tx represents cases of diagnostic curetting, lost in follow up**

	Primary Tumors			Metastases		
	E-cadherin Preserved (score 3+) (n=48)	E-cadherin Reduced (score 0-2+) (n=39)	P	E-cadherin Preserved (score 3+) (n=9)	E-cadherin Reduced (score 0-2+) (n=17)	P
Stage#						
I	31(64.6%)	24(61.5%)		-	-	
II	7(14.6%)	5(12.8%)		-	-	
III	5(10.4%)	6(15.4%)		-	-	
IV	0	1(2.6%)		-	-	
Tx†	5(10.4%)	3(7.7%)	0.843	-	-	-
Grade#						
1	14(29.2%)	8(20.5%)		5(55.6%)	1(5.8%)	
2	26(54.2%)	22(56.4%)		4(44.4%)	8(47.1%)	
3	8(16.6%)	9(23.1%)	0.574	0	8(47.1%)	**0,003***
ERα						
Preserved (score 4-12)	33(67.3%)	16(32,7%)		6(50%)	6(50%)	
Reduced (score 0-3)	15(39.5%)	23(60.5%)	**0.010***	3(21.4%)	11(78.6%)	0.218

3.1.2 Snail immunoreactivity in primary and metastatic endometrial cancer

Positive Snail immunoreactivity was seen in 28.7% of primary tumor specimens (**Table 3-1**) but no association was seen between Snail positive immunoreactivity and

tumor grade or tumor stage. No statistical correlation was observed between positive Snail immunoreactivity and reduced E-cadherin immunoreactivity but there was a statistically significant correlation between positive Snail immunoreactivity and reduced ERα immunoreactivity in primary tumors of EECs (p=0.047) (**Table 3-3**).
53.8% of the metastases of EECs showed a positive staining for Snail (**Table 3-1**). Positive Snail immunoreactivity in metastatic lesions was significantly correlated to a higher tumor grade (p=0.003) and to reduced E-cadherin immunoreactivity (p=0.003) whereas no association was observed with reduced ERα immunoreactivity (**Table 3-4**). In both, primary tumors and metastases, stromal cells were detected showing a positive immunoreactivity for Snail.

Table 3-3: Statistical correlation between Snail immunoreactivity and histopathological and immunohistochemical features in primary tumors of EECs.
***statistically significant (p<0.05); #Staging and grading according to UICC classification [134, 138]; †Tx represents cases of diagnostic curetting, lost in follow up**

	Snail negative (n=62)	Snail positive (1-5% of tumor cells) (n=15)	Snail positive (>5% of tumor cells) (n=10)	P
Stage#				
I	42(67.7%)	7(46.7%)	6(60.0%)	
II	6(9.7%)	5(33.3%)	1(10.0%)	
III	9(14.5%)	1(6.7%)	1(10.0%)	
IV	1(1.6%)	0	0	
Tx†	4(6.5%)	2(13.3%)	2(20.0%)	0.264
Grade#				
1	18(29.0%)	2(13.3%	2(20.0%)	
2	35(56.5%)	10(66.7%)	3(30.0%)	
3	9(14.5%)	3(20.0%)	5(50.0%)	0.104

E-cadherin				
Preserved (score 3+)	34(54.8%)	8(53.3%)	6(60.0%)	
Reduced (score 0, 1+, 2+)	28(45.2%)	7(46.7%)	4(40.0%)	
				>0.999
ERα				
Preserved (score 4-12)	37(59.7%)	10(66.7%)	2(20.0%)	
Reduced (score 0-3)	25(40.3%)	5(33.3%)	8(80.0%)	
				0.047*

Table 3-4: Statistical correlation between Snail immunoreactivity and histopathological and immunohistochemical features in metastases of EECs.
***statistically significant (p<0.05); #Grading according to UICC classification [134, 138]**

	Snail negative (n=12)	Snail positive (n=14)	P
Grade#			
1	5(41.7%)	1(7.1%)	
2	7(58.3%)	5(35.7%)	
3	0	8(57.2%)	
			0.003*
E-cadherin			
Preserved (score 3+)	8(66.7%)	1(7.1%)	
Reduced (score 0, 1+, 2+)	4(33.3%)	13(92.9%)	
			0.003*

ERα			
Preserved (score 4-12)	4(33.3%)	7(50.0%)	
Reduced (score 0-3)	8(66.7%)	7(50.0%)	
			0.453

3.1.3 ERα immunoreactivity in primary and metastatic endometrial cancer

ERα immunoreactivity was preserved (score 4-12) in 43.7% of primary endometrial cancers and in 57.7% of the metastatic lesions. 56.3% of primary tumors showed reduced ERα immunoreactivity (score 0-3) (**Table 3-1**). As mentioned above, reduced ERα immunoreactivity in primary tumors was significantly associated with reduced E-cadherin immunoreactivity and positive Snail immunoreactivity (**Tables 3-2 and 3-3**). Additionally, a significant correlation between reduced ERα immunoreactivity and a higher tumor grade ($p<0,001$) in the primary tumors of EECs was observed. 42.3% of the metastases showed reduced levels of ERα but with no correlation to reduced E-cadherin immunoreactivity, positive Snail immunoreactivity, or higher tumor grade.

3.1.4 Western Blot analysis of E-cadherin, Snail, ERα, and MTA3 expression with protein lysates from FFPE tissues

To analyze E-cadherin, Snail, and ERα immunoreactivities in the same tissue areas, Western Blots with proteins extracted from formalin-fixed, paraffin-embedded tissues were performed, using a recently established technique [8]. In addition, this method made it possible to analyze MTA3 expression in human tissues. The MTA3 antibody used in this study did not work for immunohistochemistry, although different antibody dilutions, antigen retrieval procedures and blocking procedures were tested (data not shown). 5 ERα positive and 6 ERα negative primary tumors were selected for this analysis. **Figure 3-2** shows the result. The Western Blot for ERα confirmed the immunohistochemical results, i.e. all 5 ERα immunohistochemically positive cases showed a clear band at appr. 67 kDa; for the 6 immunohistochemically negative cases only one (#6) showed a very faint band. E-cadherin is often seen in this series of ERα positive primary tumors. Snail expression may be associated - at least in some cases - with reduced ERα expression. These results are in line with the immunohistochemical results. Unexpectedly, MTA3 was found to be expressed in every tumor analyzed, independently of ER status. In one case (#6) a smaller variant of the MTA3 protein was observed in the Western Blot while the intensity of the correct band was reduced. The functional significance of this abnormal MTA3 variant is not clear at present.

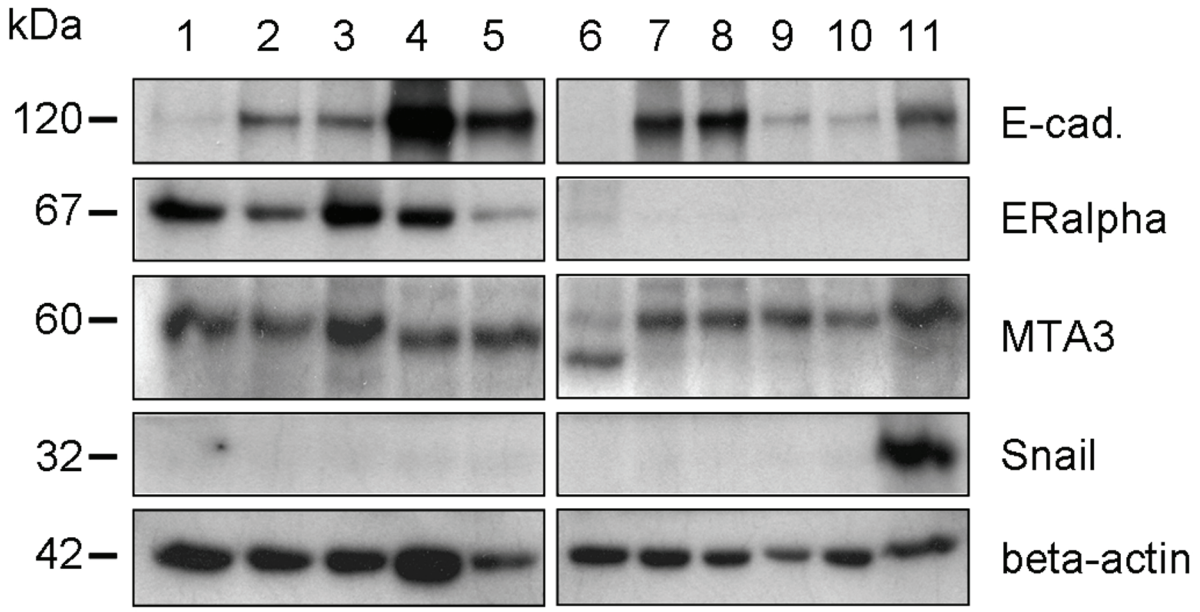

Figure 3-2: Western Blot analysis of E-cadherin, Snail, MTA3 and ERα expression in the same tissue areas of 11 formalin-fixed, paraffin-embedded primary endometrial cancers. Equal volumes (10µl) of protein extracts, as protein quantification was not practicable due to high amounts of SDS in the sample buffer, were separated by 10% SDS-PAGE and analyzed using monoclonal antibodies against E-cadherin, Snail, MTA3 and ERα. Molecular weights of the proteins are indicated. Loading of total protein was analyzed by Western Blot analysis of β-actin. The 11 formalin-fixed, paraffin-embedded tissues for protein sample preparation were selected according to ERα immunohistochemistry (5 positive – left panel and 6 negative cases – right panel).

3.1.5 Expression of E-cadherin, Snail, MTA3 and ERα in endometrial adenocarcinoma cell lines

To analyze a functional effect of Snail, several endometrial carcinoma cell lines were characterized. The expression of E-cadherin, Snail, ERα, and MTA3 in four endometrial adenocarcinoma cell lines was analyzed by Western Blot (**Figure 3-3**). A high expression of E-cadherin was seen in Ishikawa and HEC-1-A cells, in contrast to Ishikawa ER- and HEC-1-B cells where no expression of E-cadherin was detected. In Ishikawa and HEC-1-A cells the high expression levels of E-cadherin were correlated to low expression levels of Snail. In Ishikawa ER- cells the loss of E-cadherin expression was correlated to high expression levels of Snail whereas HEC-1-B cells showed only a very low expression of Snail. Ishikawa was the only ERα expressing cell line. Ishikawa ER-, HEC-1-A, and HEC-1-B showed no expression of ERα. All four cell lines showed expression of the MTA3 protein without correlation to ERα expression status.

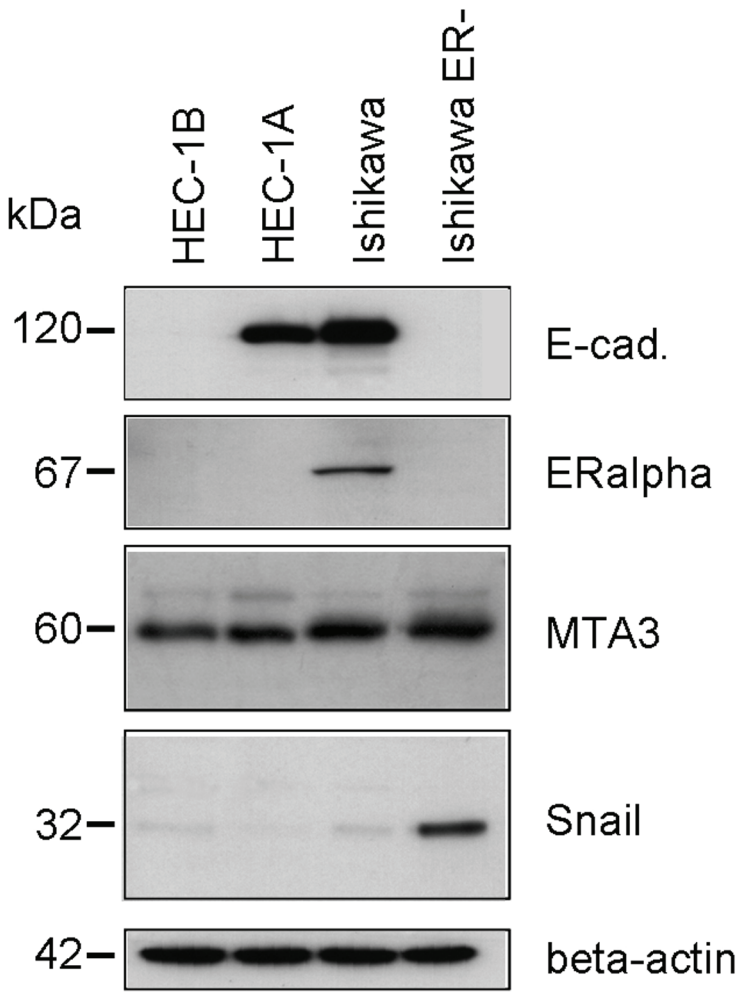

Figure 3-3: Western Blot analysis of E-cadherin, Snail, MTA3 and ERα expression. 20µg of total protein from four different endometrial cancer cell lines, Ishikawa, Ishikawa ER-, HEC-1-A and HEC-1-B, were separated by 10% SDS-PAGE and analyzed using monoclonal antibodies against E-cadherin, Snail, MTA3 and ERα. Molecular weights of the proteins are indicated. Loading of total protein was analyzed by Western Blot analysis of β-actin.

3.1.6 Wound-healing assay with Ishikawa and Ishikawa ER- cells

Cell migration is important for tumor cell invasion. As Ishikawa and its estrogen receptor negative variant Ishikawa ER- cells show an opposed expression profile of E-cadherin and Snail, the motility of these cells was analyzed by using a traditional wound-healing assay. The results shown in **Figure 3-4** are representative for three independent wound-healing assays. Both cell lines showed a visible increase in cell density over time. 24h and 48h after scraping the wound in the cell layer of the Ishikawa cells narrows a little because of the increasing cell density but even 48h after scraping no cells have migrated into the wound (**Figure 3-4A**). In contrast, the Ishikawa ER- cells started to migrate into the wound 24h after scraping the cell layer. After 48h, the wound is almost closed due to the migration of the Ishikawa ER- cells (**Figure 3-4B**). Quantification (Material and Methods **2.7**) showed that after 24h about 92 Ishikawa ER- cells and after 48h approximately 284 Ishikawa ER- cells had migrated into the wound.

Additionally a growth curve was performed for both cell lines which showed a similar proliferation rate for Ishikawa and Ishikawa ER- cells (data not shown). This confirmed that the differences in the wound-healing assay are due to a different migration potential of the cells and not because of a higher proliferation rate of Ishikawa ER- cells.

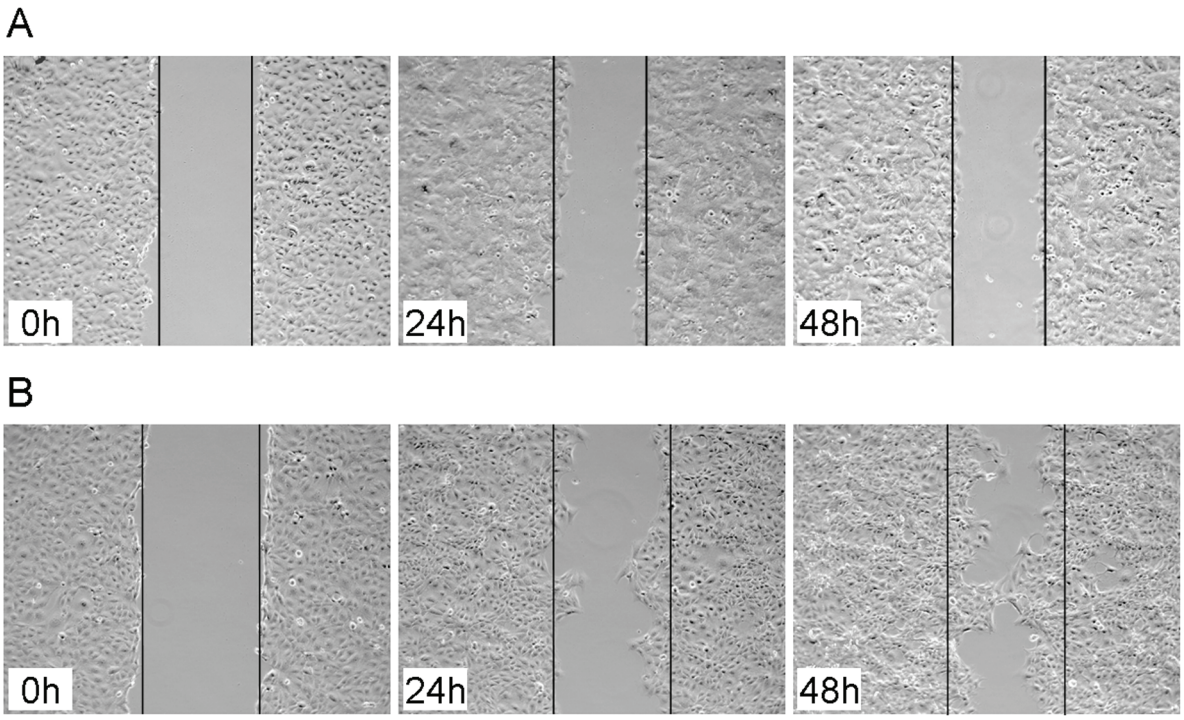

Figure 3-4: Confluent cell monolayers of Ishikawa (A) and Ishikawa ER- (B) cells were subjected to a wound-healing assay to monitor cell motility. Triplicate wells of Ishikawa or Ishikawa ER- cells were grown 24 h without serum before wounding to minimize cell proliferation. Wounding was performed, still in serum-free medium, using a standard 200µl pipette tip. The wounded areas were marked and cells were photographed at the time points indicated. This figure shows a typical result of wound closure ability of cells displaying a very low Snail expression (Ishikawa) versus cells with high protein levels of Snail (Ishikawa ER-). (magnification x100)

3.1.7 Knock down of ERα in Ishikawa cells by siRNA

Snail expression levels are supposed to be regulated indirectly through ERα signaling via MTA3 expression. To further analyze this regulatory pathway I examined silencing of ERα in the endometrial adenocarcinoma cell line Ishikawa by RNA interference with a commercially available siRNA (Ambion). This pre-designed siRNA targets the coding region of ERα mRNA as described in Material and Methods (**2.8**). In all transfection experiments cells were transfected with target specific siRNA and a non-targeting control siRNA as a negative control.
Silencing was monitored on protein level from day 3 to day 7 after transfection of siRNAs. **Figure 3-5** shows a representative Western Blot for two independent siRNA transfection assays in Ishikawa cells. Silencing of ERα by transfection of the target specific siRNA was successful as protein expression was markedly reduced at day 3

and 4 after transfection. ERα protein levels slowly increased again from day 5 to day 7 after transfection. No reduction in the protein levels of MTA3 as well as no increase in Snail protein levels was seen in Ishikawa cells transfected with ERα-specific siRNA compared to Ishikawa cells transfected with control siRNA. Moreover, the reduction of ERα protein levels had no effect on the expression of E-cadherin.

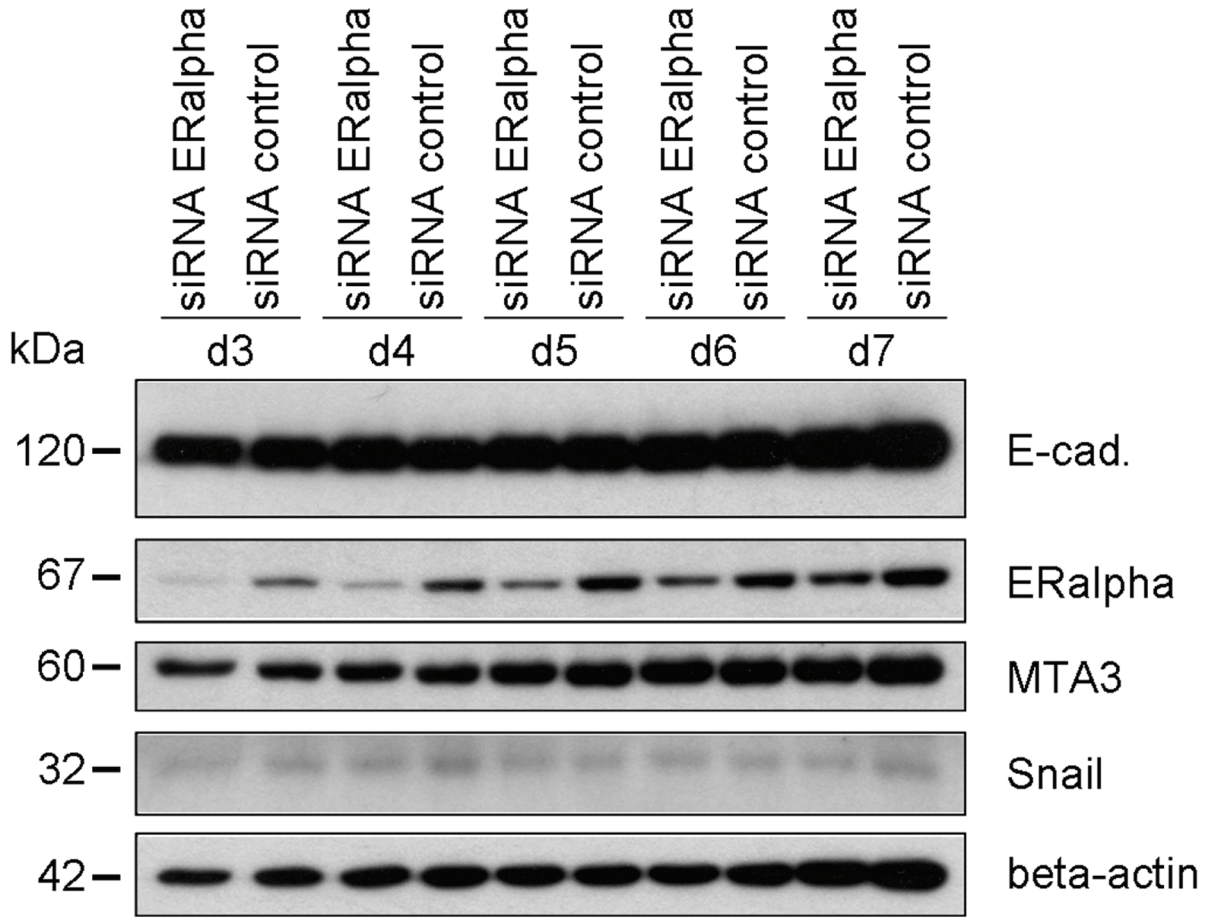

Figure 3-5: Western Blot analysis of E-cadherin, Snail, MTA3 and ERα expression in Ishikawa cells days 3 to 7 after transfection with ERα specific siRNA and non-specific control siRNA, respectively. 15µg of total protein were separated by 10% SDS-PAGE and analyzed using monoclonal antibodies against E-cadherin, Snail, MTA3 and ERα. Molecular weights of the proteins are indicated. Loading of total protein was analyzed by Western Blot analysis of β-actin.

3.1.8 Treatment of Ishikawa cells with the ER antagonist fulvestrant ('Faslodex')

Another way to modulate ERα signaling is the treatment of cells with so called selective estrogen receptor modulators (SERMs), which have agonist and/or antagonist functions depending on the target promoters and tissues. Fulvestrant ('Faslodex') is a pure steroidal anti-estrogen without displaying partial agonist activity. Fulvestrant binds to ER monomers thereby inhibiting receptor dimerization and

functional activation. Instead of being translocated to the nucleus degradation of the ER is accelerated. To investigate the effect of fulvestrant on ERα and the putative regulatory pathway of Snail expression via ERα signaling and MTA3, Ishikawa cells were treated with 1µM fulvestrant for 6 days. The effect of fulvestrant treatment was monitored on protein level from day 3 to day 6 after the application of the ER antagonist. **Figure 3-6** shows a representative Western Blot for two independent fulvestrant treatment assays in Ishikawa cells. ERα protein levels are considerably reduced in Ishikawa cells treated with fulvestrant compared to untreated cells, reflecting the increased degradation of ERα. Despite the antiestrogenic effects of fulvestrant neither a reduction of MTA3 nor an increase of Snail could be detected on the protein level. E-cadherin protein expression was unaffected by fulvestrant treatment as well.

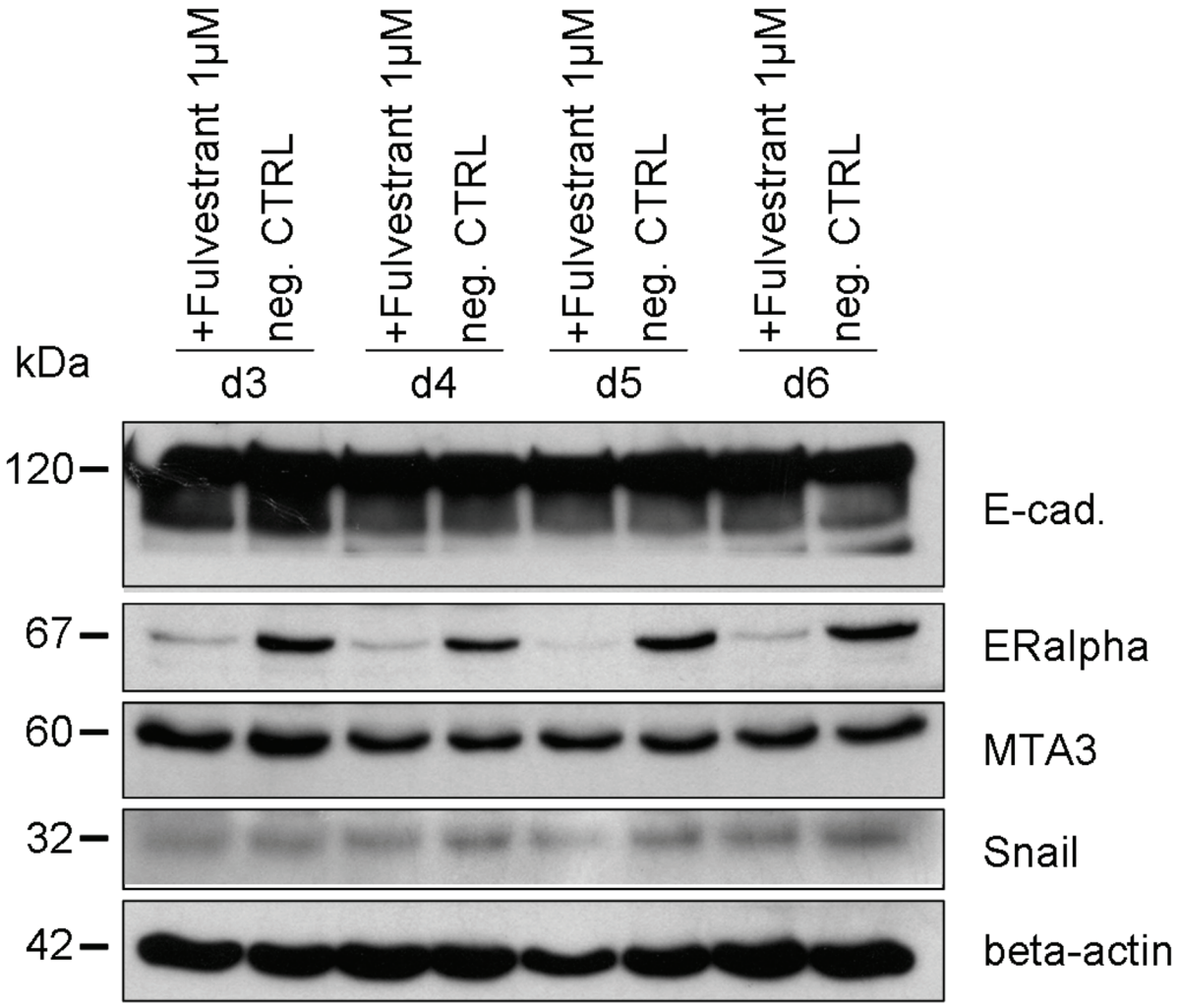

Figure 3-6: Western Blot analysis of E-cadherin, Snail, MTA3 and ERα expression in Ishikawa cells. Cells were treated as described in "Material and Methods" with 1µM fulvestrant or control (neg. CTRL) containing media for the indicated times. 20µg of total protein were separated by 10% SDS-PAGE and analyzed using monoclonal antibodies against E-cadherin, Snail, MTA3 and ERα. Molecular weights of the proteins are indicated. Loading of total protein was analyzed by Western Blot analysis of β-actin.

3.1.9 Wound-healing assay with untreated Ishikawa cells and Ishikawa cells treated with fulvestrant

Although no differences were found in the protein expression profile of MTA3, Snail and E-cadherin in fulvestrant-treated Ishikawa cells compared to untreated cells, it was analyzed if there is an effect of the ER antagonist on the migratory potential of the cells. Therefore a traditional wound-healing assay was performed with fulvestrant-treated Ishikawa cells compared to untreated ones. The results shown in **Figure 3-7** are representative for three independent wound-healing assays. 1µM fulvestrant was added to the Ishikawa cells 4 days before scraping the wound. At this time point it had been shown by Western Blot analysis that ERα protein levels were markedly down-regulated. Fulvestrant-treated as well as untreated Ishikawa cells showed a visible increase in cell density over time. Nevertheless neither the fulvestrant-treated (**Figure 3-7A**) nor the untreated Ishikawa cells (**Figure 3-7B**) had migrated into the wound 24h or 48h after scraping. No differences could be observed in the migratory potential of fulvestrant-treated compared to untreated Ishikawa cells.

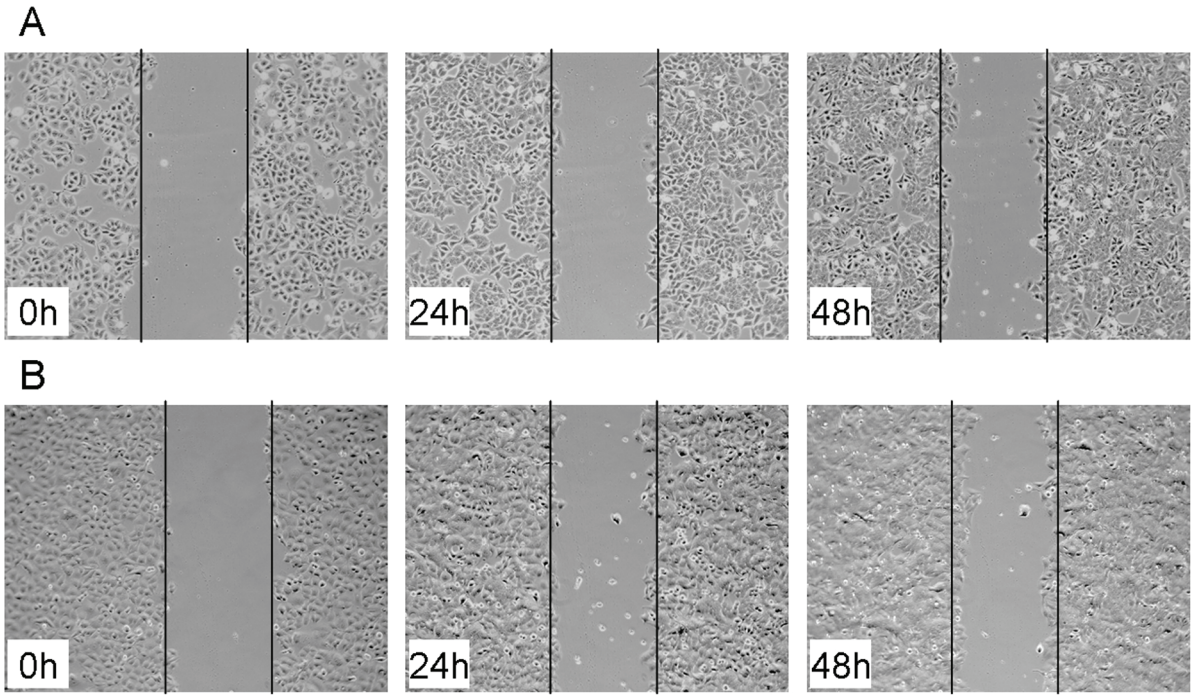

Figure 3-7: Confluent cell monolayers of Ishikawa cells were subjected to a wound-healing assay to monitor cell motility. Cells were treated as described in "Material and Methods" with 1µM fulvestrant (A) or control (B) containing media. Triplicate wells of Ishikawa cells, fulvestrant-treated or untreated, were grown 24 h without serum before wounding to minimize cell proliferation. Wounding was performed, still in serum-free medium, using a standard 200µl pipette tip. The wounded areas were marked and cells were photographed at the time points indicated. There were no detectable differences in the migration potential of fulvestrant-treated and untreated Ishikawa cells. (magnification x100)

3.1.10 Long time treatment of Ishikawa cells with 17β-estradiol

Ligand binding is known to modulate ER levels by altering transcription and stability of the resulting mRNA and the turnover of the protein. For the breast cancer cell line MCF7 it was shown [109] that exposure to estrogens cause a decrease of ER mRNA and protein levels indicating a feedback inhibition of steady-state ER levels as a primary response to an estrogenic stimulus. To analyze the regulation of ERα protein expression in Ishikawa cells by 17β-estradiol, also regarding the protein expression levels of MTA3, Snail and E-cadherin, a long time treatment of Ishikawa cells with 1μM 17β-estradiol was performed for 8 days. The response to the estrogen exposure was monitored on protein level from day 4 to day 8 after the application of 17β-estradiol. **Figure 3-8** shows a representative Western Blot for two independent long time stimulation assays with 17β-estradiol in Ishikawa cells. ERα protein levels were markedly reduced in estrogen stimulated cells when compared to control cells. No differences were found in the protein expression of MTA3 in treated and untreated Ishikawa cells, respectively. Nevertheless, Snail protein levels showed an increase over time in the 17β-estradiol-treated Ishikawa cells. This change in protein expression was not seen in control cells. However, E-cadherin protein levels remained unaffected by estrogen stimulation and increasing cellular Snail protein levels. There were no differences in the expression of E-cadherin in 17β-estradiol treated or untreated Ishikawa cells.

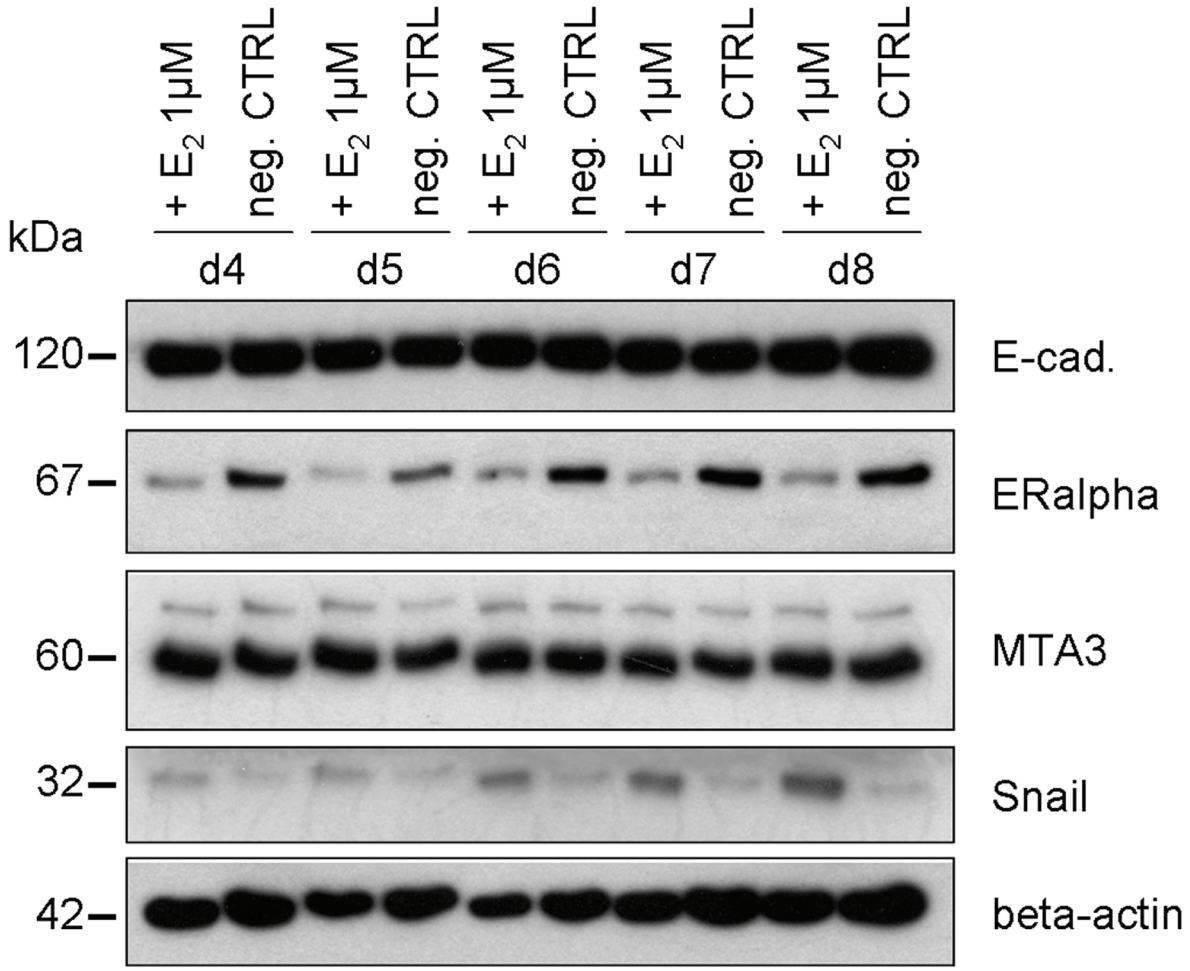

Figure 3-8: Western Blot analysis of E-cadherin, Snail, MTA3 and ERα expression in Ishikawa cells. Cells were treated as described in "Material and Methods" with 1µM 17β-estradiol (E_2) or control (neg. CTRL) containing media for the indicated times. 20µg of total protein were separated by 10% SDS-PAGE and analyzed using monoclonal antibodies against E-cadherin, Snail, MTA3 and ERα. Molecular weights of the proteins are indicated. Loading of total protein was analyzed by Western Blot analysis of β-actin.

3.2 E-CADHERIN AND ITS REPRESSOR SNAIL IN OVARIAN CANCER

A total of 48 specimens of primary tumors and 50 specimens of metastases of ovarian carcinomas were examined for E-cadherin and Snail immunoreactivity, of which 47 cases were matched pairs of primary tumors and their corresponding metastases (**Figure 3-9**). A summary of the clinicopathological characteristics of the patients is given in **Table 3-5**.

Table 3-5: Clinicopathological characteristics of the ovarian cancer patients. *includes 3 mucinous, 2 Muellerian, 1 Brenner and 1 clear cell

Characteristic	n	%
Age, years		
Median	63	
Range	29 - 82	
Histological grade		
1	3	6
2	11	22
3	37	72
FIGO stage		
III	39	76
IV	12	24
TNM staging		
T		
1 (1a+1c)	3	6
2 (2b+2c)	2	4
3 (3b+3c)	46	90
N		
0	6	12
1	22	43
x	23	45
M		
0	38	74
1	11	22
x	2	4
Histological type		
Serous	39	76
Endometrioid	5	10
Others*	7	14
End state		
Dead	31	61
Alive	20	39

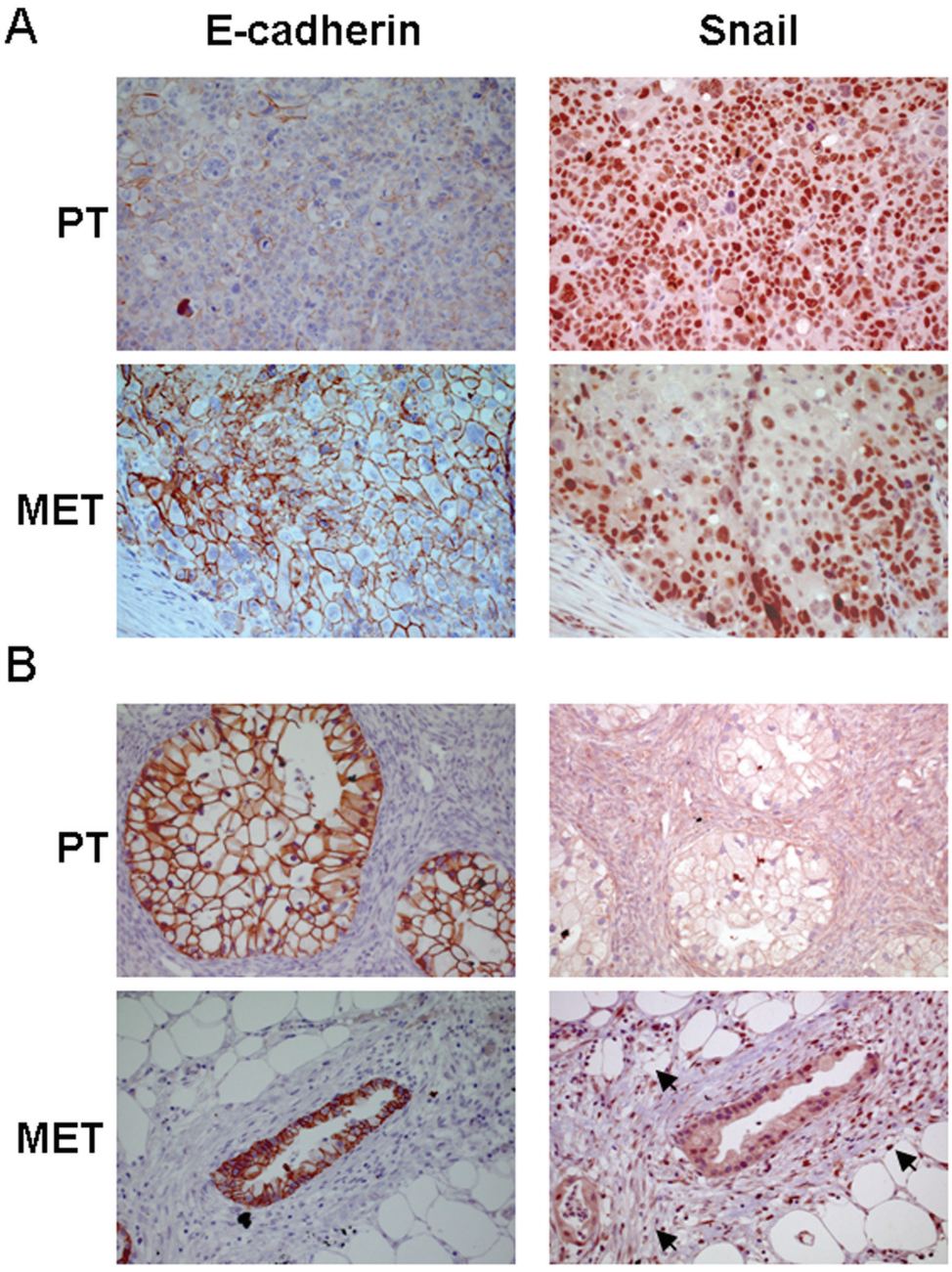

Figure 3-9: (A, B) Consecutive immunostainings for E-cadherin and Snail of two ovarian cancer cases, showing the primary tumors and the corresponding metastases.
A: An ovarian cancer case showing reduced E-cadherin immunostaining in the primary tumor, while E-cadherin immunoreactivity is preserved in the corresponding metastasis. Snail immunoreactivity is positive for both, primary tumor and metastasis (immunoperoxidase staining, x200). B: An ovarian cancer case showing preserved E-cadherin immunostaining in primary tumor and corresponding metastasis, respectively. Snail immunoreactivity is negative in the primary tumor whereas it is positive in the corresponding metastasis. Note that also tumor-associated stromal cells show nuclear staining for Snail (indicated by arrow heads) (immunoperoxidase staining, x200).
PT, primary tumor; MET, corresponding metastasis

3.2.1 E-cadherin immunoreactivity in primary and metastatic ovarian cancer

E-cadherin immunoreactivity was preserved (Score 3+) in 75% of primary ovarian cancers and 78% of the corresponding metastases. A reduced E-cadherin immunoreactivity (Score 0, 1+, 2+) was found in 25% of primary tumors and 22% of metastases (**Table 3-6**).
There was a significant correlation between E-cadherin immunoreactivity in ovarian cancers and their corresponding metastases ($p<0.001$). Out of 35 primary tumors with preserved E-cadherin immunoreactivity, 33 (94.3%) also showed preserved immunoreactivity in the corresponding metastases. Out of 12 primary tumors with reduced E-cadherin immunoreactivity, 8 (66.7%) also showed reduced immunoreactivity in the corresponding metastases (**Table 3-7**).
No association was found between E-cadherin immunoreactivity and clinicopathological factors including patient age, tumor grade, histological subtype, and FIGO-stage in primary ovarian cancers, nor in the corresponding metastases.

Table 3-6: E-cadherin and Snail immunoreactivity in primary ovarian cancer and corresponding metastases.
*** for n=47 cases, matched pairs of primary tumors and their corresponding metastases were available for analysis; n, number of patients**

	Primary Tumors (n=48)*		Metastases (n=50)*	
E-cadherin	**(n)**	**(%)**	**(n)**	**(%)**
Reduced (score 0, 1+, 2+)	12	25.0	11	22.0
Preserved (score 3+)	36	75.0	39	78.0
Snail	**(n)**	**(%)**	**(n)**	**(%)**
Positive	18	37.5	26	52.0
Negative	30	62.5	24	48.0

3.2.2 Snail immunoreactivity in primary and metastatic ovarian cancer

Positive immunoreactivity for Snail was detected in 37.5% of primary ovarian cancers and in 52% of corresponding metastases (**Table 3-6**). There was a significant correlation between Snail immunoreactivity in ovarian cancers and their corresponding metastases ($p<0.001$). Out of 17 primary tumors with positive Snail immunoreactivity, 16 (94.1%) also showed Snail immunoreactivity in the corresponding metastases. Out of 30 primary tumors with negative Snail immunoreactivity, 21 (70.0%) were also negative for Snail in the corresponding

metastases (**Table 3-7**). No association was found between Snail immunoreactivity and clinicopathological factors including patient age, tumor grade, histological subtype, and FIGO-stage in primary ovarian cancers, nor in the corresponding metastases.
There was no statistically significant correlation between E-cadherin and Snail immunoreactivity, neither in primary ovarian cancers nor in their metastases. Out of 36 primary tumors with preserved E-cadherin immunoreactivity, 13 (36.1%) showed positive Snail and 23 (63.9%) negative Snail immunoreactivity (p=0.743). Out of 39 metastases with preserved E-cadherin immunoreactivity, 21 (53.8%) showed positive Snail and 18 (46.2%) negative Snail immunoreactivity (p=0.623). Occasionally tumor-associated stromal cells were observed showing nuclear immunoreactivity for Snail.

Table 3-7: Correlation of E-cadherin and Snail immunoreactivity in primary ovarian cancer compared to the corresponding metastases*.
*** n=47 cases for which both the primary tumors and their corresponding metastases were available.**
Primary, primary ovarian cancer
Metastasis, corresponding metastases of primary ovarian cancers
positive, preserved immunoreactivity for E-cadherin (3+) or Snail positive
negative, reduced immunoreactivity for E-cadherin (0, 1+, 2+) or Snail negative

	E-cadherin (n)	**Snail** (n)
Primary positive	35	17
Primary negative	12	30
Primary positive, Metastasis positive	33 (94.3% of n=35)	16 (94.1% of n=17)
Primary positive, Metastasis negative	2 (5.7% of n=35)	1 (5.9% of n=17)
Primary negative, Metastasis negative	8 (66.7% of n=12)	21 (70.0% of n=30)
Primary negative, Metastasis positive	4 (33.3% of n=12)	9 (30.0% of n=30)

3.2.3 E-cadherin and Snail expression and overall survival

There were significant differences in patients' overall survival with regard to E-cadherin expression in primary ovarian cancer ($p_{(log\text{-}rank)}$=0.008) (**Figure 3-10)**. Patients with a reduced E-cadherin immunoreactivity (n=12) had a median overall survival of 17.9 months (95% CI [5.8-30.0]) compared to 48.8 months (95% CI [24.0-73.6]) in patients with preserved E-cadherin immunoreactivity (n=36). The

corresponding hazard ratio was 2.82 (95% CI [1.3-6.3]). In metastases, no significant differences were found in overall survival when examining E-cadherin expression.
There was a borderline significant difference in the overall survival of patients with positive Snail expression in metastases of ovarian cancer compared to patients with negative Snail expression ($p_{(log\text{-}rank)}$=0.047) (**Figure 3-11)**, whereas these differences were not found in primary tumors. Patients with positive Snail immunoreactivity in their metastases (n=26) had a lower median overall survival of 17.9 months (95% CI [12.2-23.7]) with a hazard ratio of 2.10 (95% CI [1.0-4.4]). The median overall survival of patients with Snail negative metastases (n=24) has not yet been reached at 55 months of median follow-up time.

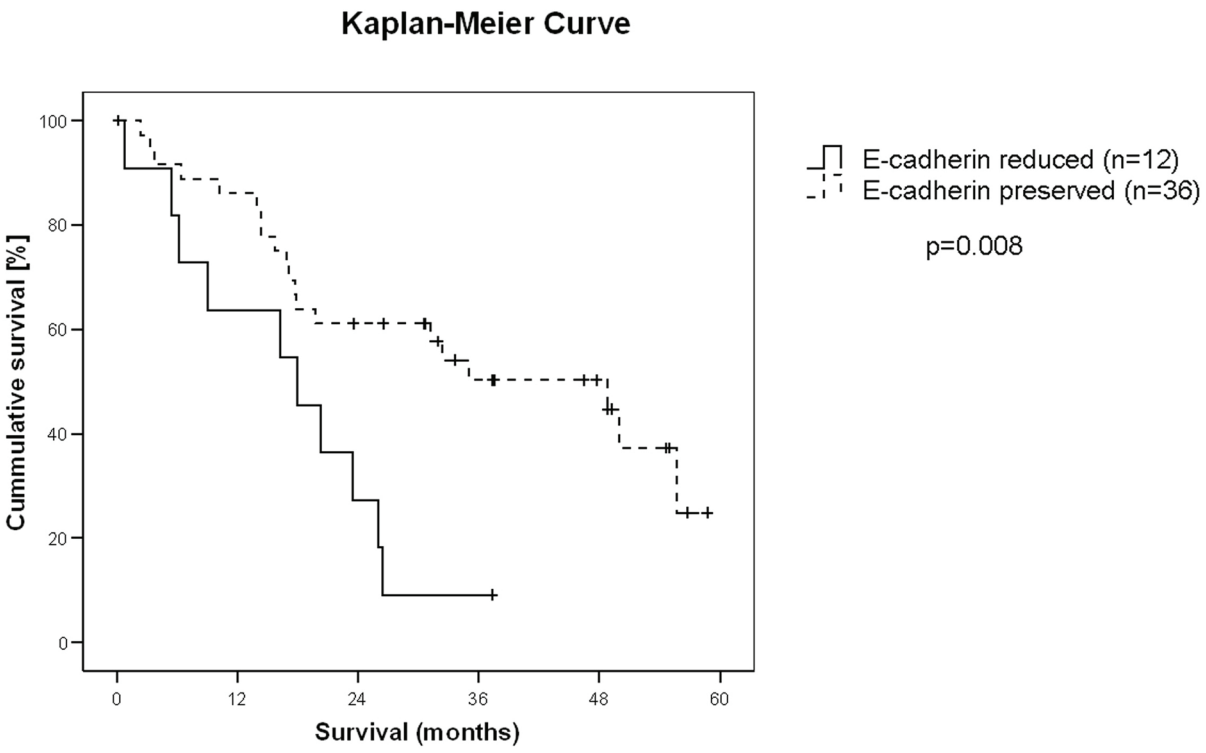

Figure 3-10: Kaplan-Meier survival curve for ovarian carcinoma patients according to E-cadherin immunoreactivity in primary ovarian cancers.
Discontinuous line represents preserved E-cadherin immunoreactivity. Continuous line represents reduced E-cadherin immunoreactivity. Patients with reduced E-cadherin immunoreactivity had a significantly shorter overall survival (p=0.008).

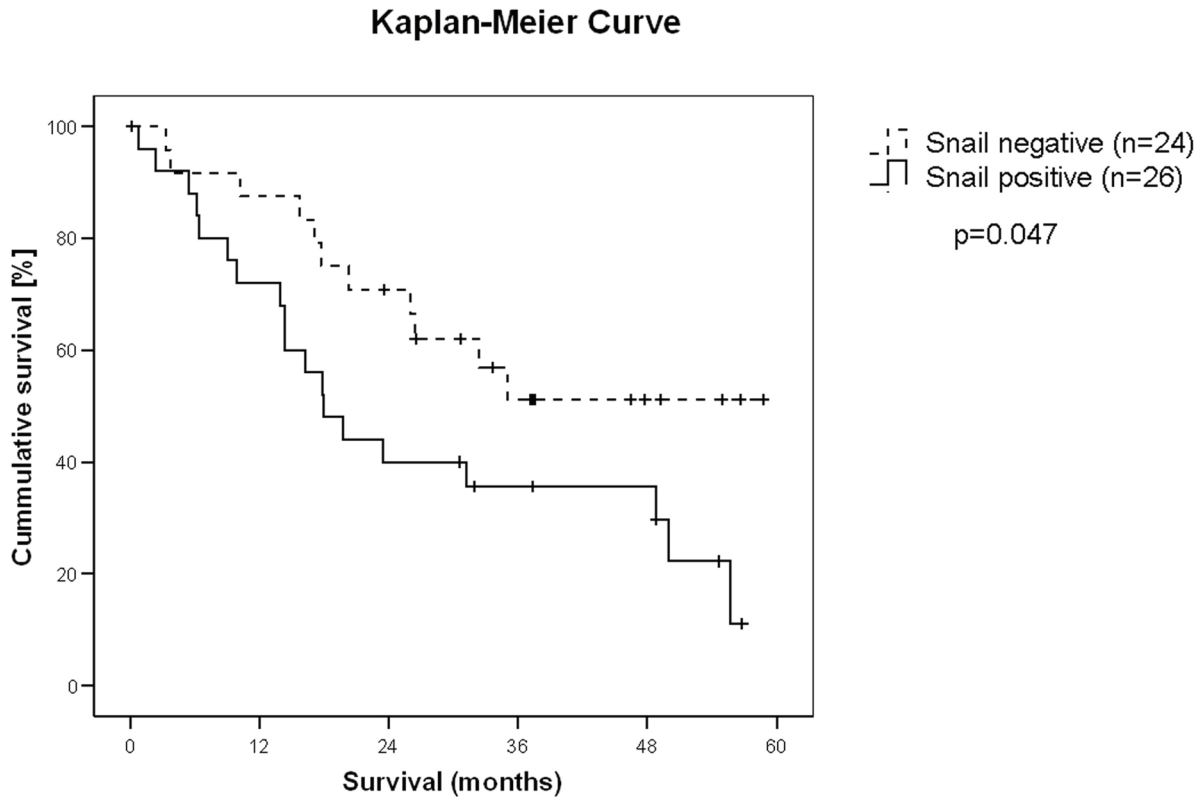

Figure 3-11: Kaplan-Meier survival curve for ovarian carcinoma patients according to Snail immunoreactivity in metastases of ovarian cancer. Discontinuous line represents negative Snail immunoreactivity. Continuous line represents positive Snail immunoreactivity. Patients with positive Snail immunoreactivity had a significantly shorter overall survival (p=0.047).

To analyze the patients' overall survival with regard to different combinations of E-cadherin and Snail expression profiles the patients were divided in four different groups which are shown in **Table 3-8A and B**. The comparison of survival between the different groups was done by Cox- proportional hazard regression. Since normal non-cancerous epithelial tissue is characterized by preserved E-cadherin and negative Snail expression, this expression profile served as a reference standard. When analyzing the protein expression profile in primary ovarian tumors with regard to patient survival, only patients with the expression profile "E-cadherin reduced and Snail positive" showed a significantly (p=0.002) higher risk for the occurrence of death with a hazard ratio of 5.98 (95% CI [1.9-18.0]) when compared to the reference group (**Table 3-8A**). In case of the corresponding metastases, a significantly (p=0.022) higher risk for the occurrence of death was seen in patients with the same expression profile of "E-cadherin reduced and Snail positive" with a hazard ratio of 4.36 (95% CI [1.2-15.4]) when compared to the reference group (**Table 3-8B**).

Table 3-8A: Comparison of survival in patient groups with different expression profiles of E-cadherin and Snail in primary ovarian cancer using Cox- proportional hazard regression.
[#] was set as the reference group; * statistically significant (p<0.05)
+, preserved immunoreactivity for E-cadherin (3+) or Snail positive
-, reduced immunoreactivity for E-cadherin (0, 1+, 2+) or Snail negative
HR, hazard ratio for the occurrence of death; CI, confidence interval; n, number of patients

Primary ovarian cancer (n=48)	p	HR	95% CI for HR	
			Lower	Upper
Ecadherin+/Snail- (n=23)[#]		1.00		
Ecadherin+/Snail+ (n=13)	0.590	1.28	0.521	3.149
Ecadherin-/Snail- (n=7)	0.166	2.12	0.731	6.155
Ecadherin-/Snail+ (n=5)	**0.002***	5.98	1.981	18.043

Table 3-8B: Comparison of survival in patient groups with different expression profiles of E-cadherin and Snail in metastases of ovarian cancer using Cox- proportional hazard regression.
[#] was set as the reference group; * statistically significant (p<0.05)
+, preserved immunoreactivity for E-cadherin (3+) or Snail positive
-, reduced immunoreactivity for E-cadherin (0, 1+, 2+) or Snail negative
HR, hazard ratio for the occurrence of death; CI, confidence interval; n, number of patients

Metastases of ovarian cancer (n=50)	p	HR	95% CI for HR	
			Lower	Upper
Ecadherin+/Snail- (n=18)[#]		1.00		
Ecadherin+/Snail+ (n=21)	0.077	2.26	0.917	5.546
Ecadherin-/Snail- (n=6)	0.329	1.85	0.537	6.396
Ecadherin-/Snail+ (n=5)	**0.022***	4.36	1.232	15.417

3.3 E-CADHERIN AND ITS REPRESSOR SNAIL IN PLACENTAL TISSUE OF NORMAL, PREECLAMPTIC AND HELLP PREGNANCIES

A natural model for migration and invasion is placental development during embryo implantation. Fetally-derived extravillous trophoblast cells are supposed to undergo epithelial to mesenchymal transition and it was shown, that Snail is expressed in this cell type [121]. A total of 7 specimens of normal placenta, 7 specimens of preeclamptic placenta, and 5 specimens of HELLP syndrome placenta were examined for E-cadherin and Snail immunoreactivity. A summary of the clinical details of the cases are shown in **Table 3-9**.

Table 3-9: Clinical details on the patients and newborns in the HELLP, preeclamptic and normal control group. The data are presented as mean values.
SD: standard deviation

	HELLP	Preeclampsia	normal control
week of gestation at delivery	33.8 (SD 2.8)	33.18 (SD 0.83)	38.2 (SD 3.9)
neonatal birthweight (g)	1796.2 (SD 689.1)	1409 (SD 547)	3435 (SD 412.2)
pH umbilical artery	7.332 (SD 0.09)	7.29 (SD 0.06)	7.27 (SD 0.09)
Apgar score at 5 min	9.2 (SD 0.84)	9.36 (SD 0.67)	9.4 (SD 0.9)
Apgar score at 10 min	9.8 (SD 0.5)	9.73 (SD 0.47)	10 (SD 0)

3.3.1 Snail and E-Cadherin immunoreactivity in placental tissue

A moderate Snail immunoreactivity in extravillous trophoblast (EVT) cells was found in the decidua of normal control placentas (**Figure 3-12A**). The immunoreactivity of Snail was significantly increased in EVTs of preeclamptic placentas (**Figures 3-12B**, **3-14A**, p=0.019). The increase in Snail immunoreactivity was accompanied by a significant reduction of E-cadherin immunoreactivity in preeclamptic EVTs compared to normal control placentas (**Figures 3-13A and B**, **3-14B**, p=0.016).
An increase of Snail immunoreactivity and concomitant reduction of E-cadherin immunoreactivity was also observed in EVTs of HELLP placentas compared to normal control placentas although not statistically significant (**Figures 3-12C**, **3-13C, 3-14A and B**).
For Snail immunoreactivity the semi-quantitative immunoreactivity score (IRS) was 4.07 in normal, 6.71 in preeclamptic and 6.8 in HELLP placentas. This corresponds to an increase of Snail immunoreactivity of approximately 65% in preeclamptic and 67% in HELLP placentas. The IRS score for E-cadherin immunoreactivity was 7.29 in normal, 3.86 in preeclamptic and 5.5 in HELLP placentas. This corresponds to a

reduction of E-cadherin immunoreactivity of about 47% in preeclamptic and 25% in HELLP placentas.
Additionally, the villous trophoblast was examined for Snail and E-cadherin immunoreactivity. There was a slight increase seen for E-cadherin immunoreactivity in the villous trophoblast cells of preeclamptic and HELLP placentas compared to the normal control placenta (data not shown). A weak Snail immunoreactivity in villous trophoblast cells was observed in very few cases of normal, preeclamptic and HELLP placentas and showed a slight increase in the pathologic forms of placentas compared to normal control placenta (data not shown). The changes in Snail and E-cadherin immunoreactivity in the villous trophoblast cells of preeclamptic and HELLP placentas compared to normal control placentas showed no statistical significance.

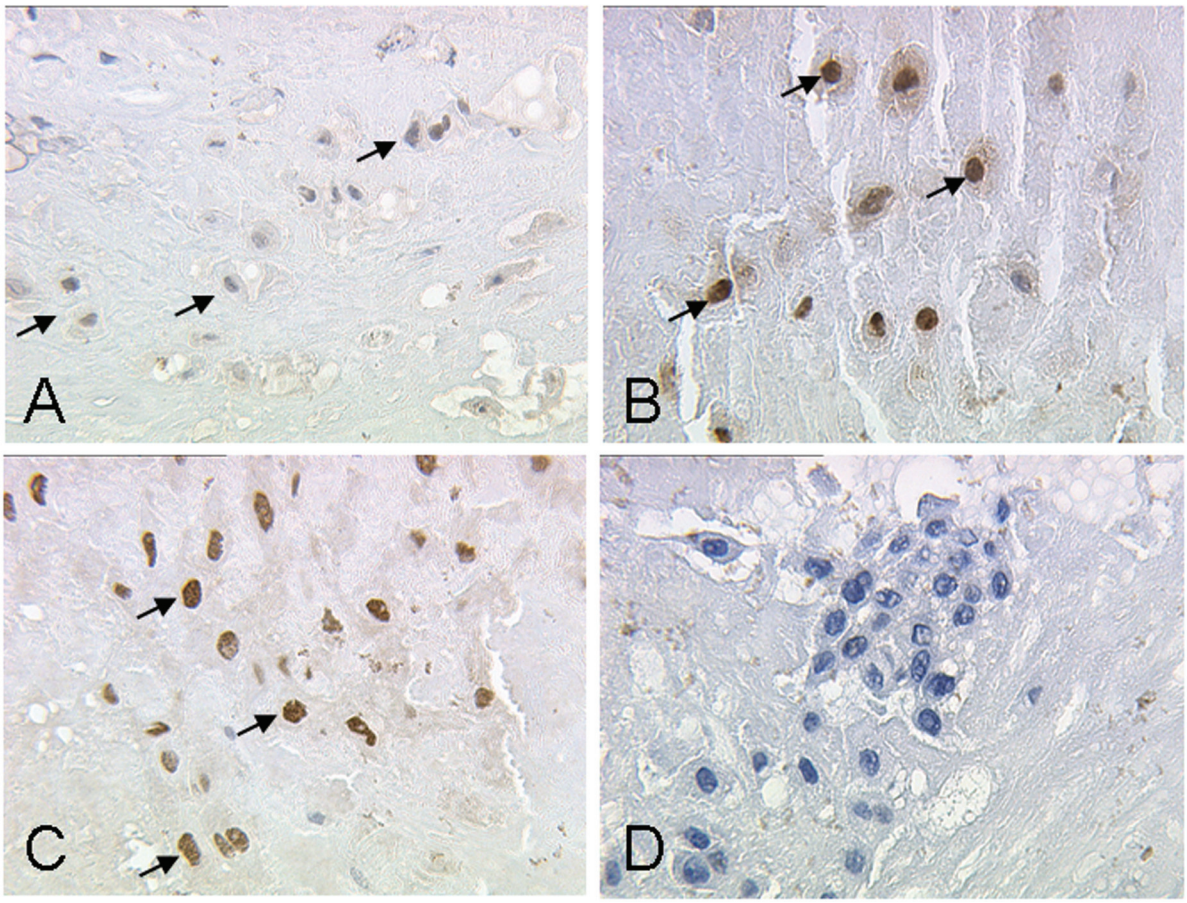

Figure 3-12: (A-D) Snail immunoreactivity in extravillous trophoblast cells (arrows)
A: normal placenta (immunoperoxidase staining, x250). B: preeclampsia (immunoperoxidase staining, x250). C: HELLP (immunoperoxidase staining, x250). D: negative control (primary antibody was replaced with pre-immune mouse serum) (immunoperoxidase staining, x250).

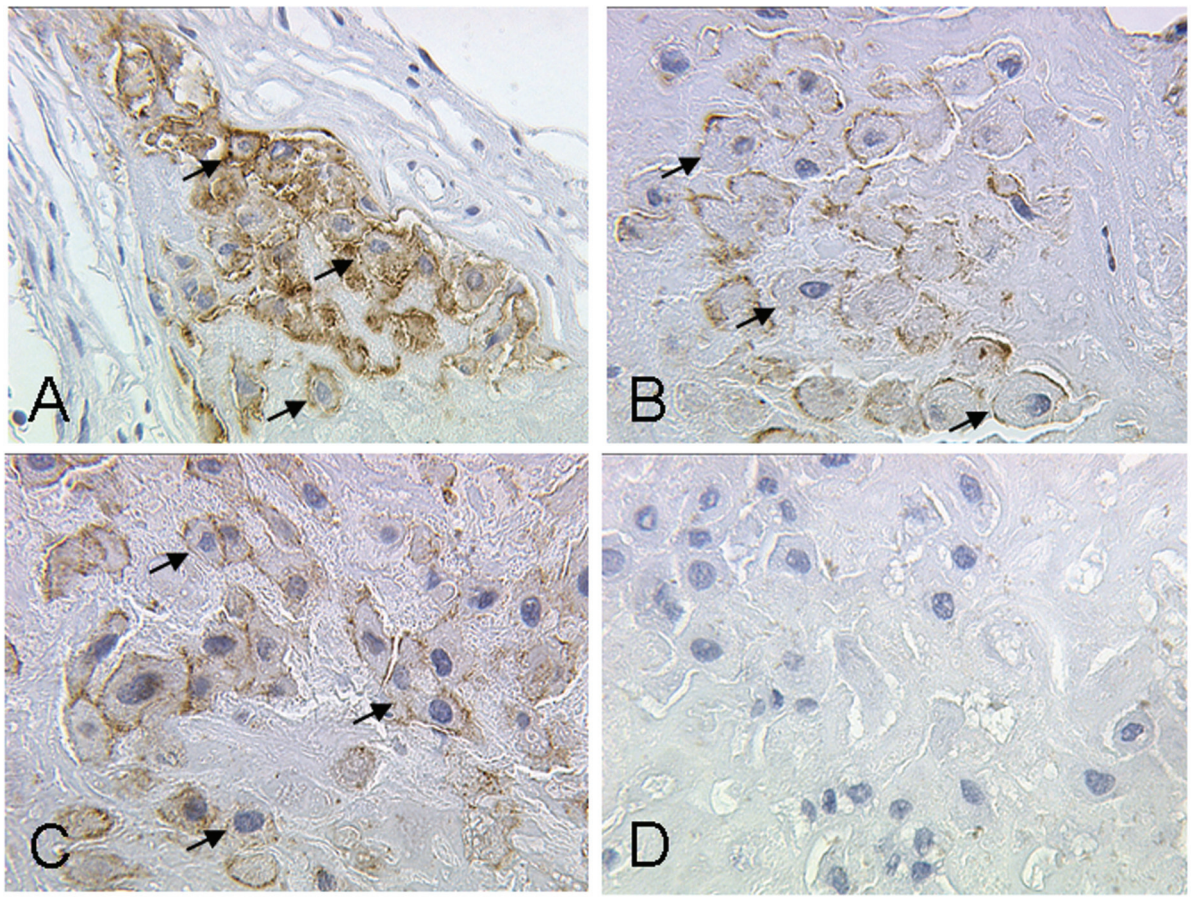

Figure 3-13: (A-D) E-cadherin immunoreactivity in extravillous trophoblast cells (arrows)
A: normal placenta (immunoperoxidase staining, x250). B: preeclampsia (immunoperoxidase staining, x250). C: HELLP (immunoperoxidase staining, x250). D: negative control (primary antibody was replaced with pre-immune mouse serum) (immunoperoxidase staining, x250).

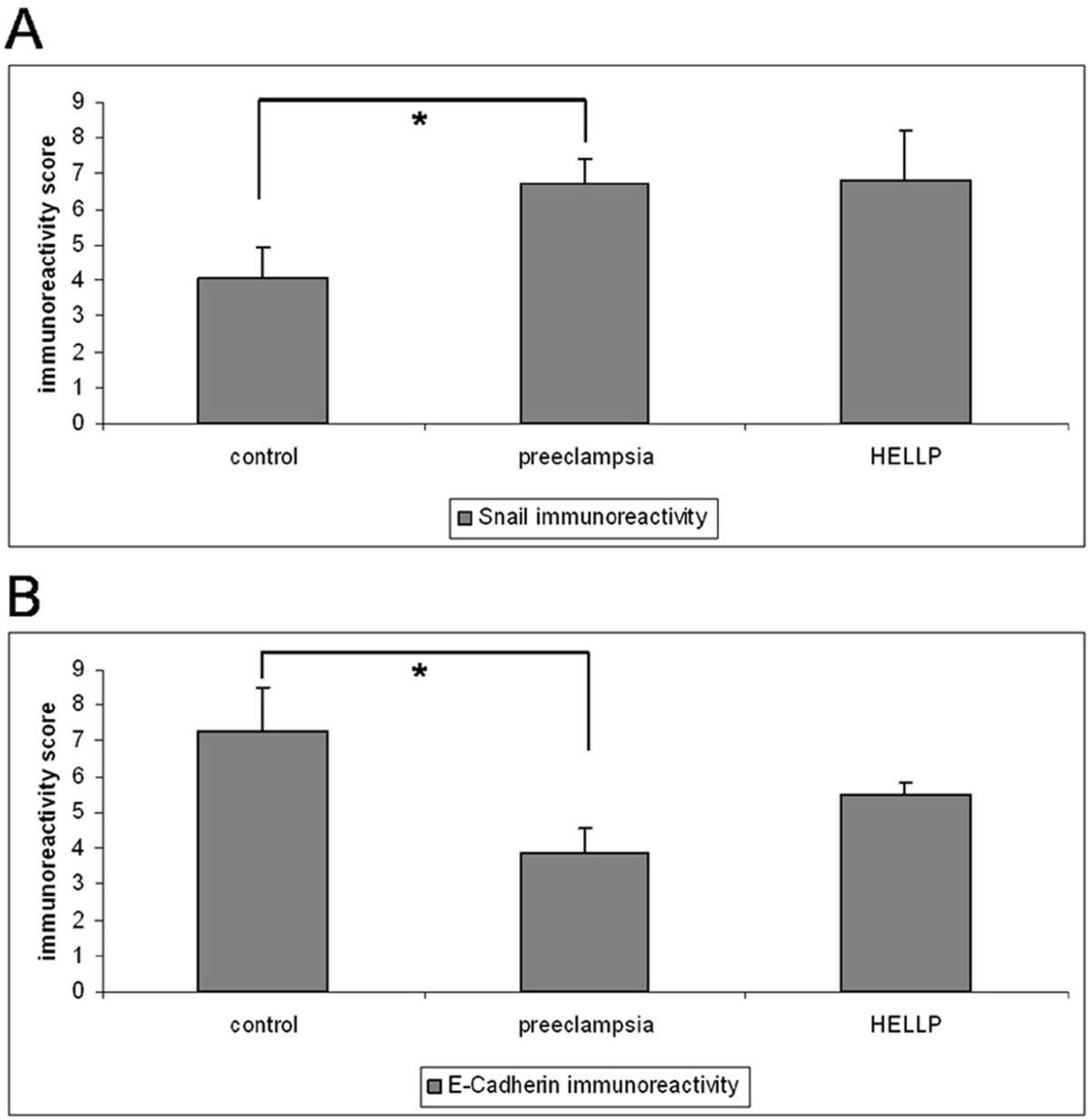

Figure 3-14: (A,B) Staining intensity of Snail and E-cadherin in normal term placenta, preeclamptic and HELLP syndrome extravillous trophoblast cells determined by the semi-quantitative immunoreactivity score on the different tissue slides. Data shown represent means ± SEM.
A: Differences in Snail immunoreactivity in normal term placenta and preeclamptic extravillous trophoblast cells (*) are statistically significant (p=0.019). B: Differences in E-cadherin immunoreactivity in normal term placenta and preeclamptic extravillous trophoblast cells (*) are statistically significant (p=0.016).

3.3.2 Characterisation of Snail and E-cadherin expressing cells in decidual tissue

To prove that the Snail- and E-cadherin-expressing cells in the decidual tissue of normal, preeclamptic and HELLP placentas are indeed EVTs, fluorescence double staining with a cell marker (cytokeratin 7 for trophoblast cells) and with either a Snail-

or an E-cadherin-specific antibody was performed. The staining with the monoclonal anti-E-cadherin antibody in combination with the Cy3-labeled anti-cytokeratin 7 antibody showed that the E-cadherin-expressing cells were positive for cytokeratin 7 (Figure **3-15A**). Cytokeratin 7 positivity was also shown for Snail-expressing cells (Figure **3-15B**). Thereby E-cadherin- and Snail-expressing cells were identified as EVTs.

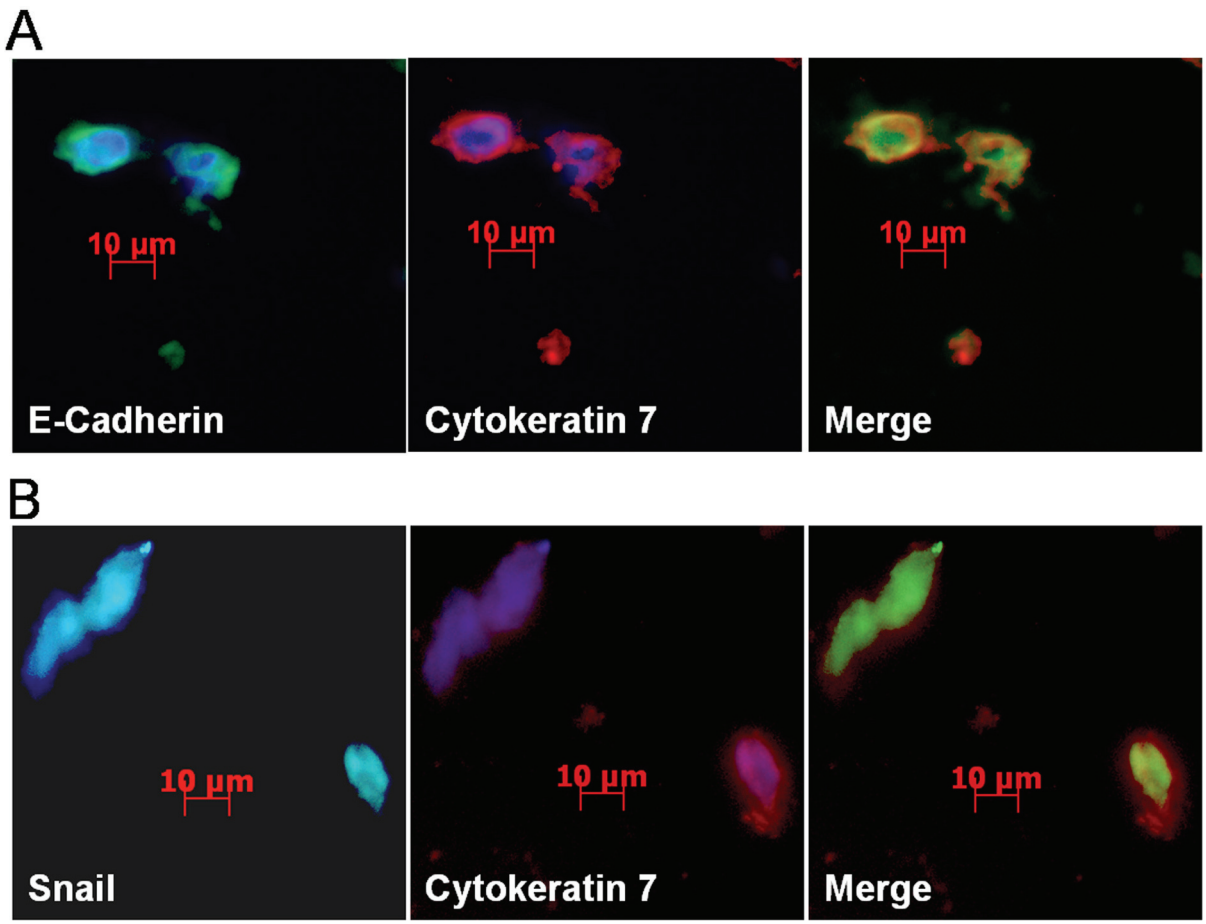

Figure 3-15: Immunofluorescence double staining in cryosections of normal control placenta.
A: Immunofluorescence double staining for cytokeratin 7 and E-cadherin. B: Immunofluorescence double staining for cytokeratin 7 and Snail.
Cy2 staining is colored in green, whereas Cy3 staining is colored in red, (magnification x400).

4 DISCUSSION

Loss of E-cadherin expression and/or function is thought to be one of the most important events during carcinoma progression [14]. During tumor progression, *E-cadherin* can be functionally inactivated by different mechanisms, including gene mutations [12], promoter hypermethylations [135, 152] and direct transcriptional repression [5, 26, 51]. The zinc-finger transcription factor Snail is one of the repressors involved in E-cadherin down-regulation and a master molecule of epithelial-mesenchymal transition (EMT) [21]. Additionally, Snail is considered as an important regulator of invasiveness during tumor progression [21]. Up to now, more than 2000 cases from at least nine different tumor types reported in more than 21 studies have been analyzed for Snail expression, including carcinomas from breast, stomach, colon, liver, ovary, oesophagus, head and neck, and synovial sarcomas [7]. These studies have provided a wealth of information on the biology of EMT in human tumors and point out a critical role for Snail in tumor dedifferentiation, at least in some types of cancer. Almost all of these studies have analyzed Snail's mRNA expression. However, mRNA levels may not correspond to the active Snail protein, which was shown to be highly unstable with a short half-life of about 25 minutes [163]. A monoclonal antibody, which was established recently in our group [121], now allows to detect the active, i.e. nuclear localized, Snail protein in formalin-fixed, paraffin-embedded specimens of human tumors. In the meantime, another group reported the establishment of another Snail-specific monoclonal antibody [39].

4.1 E-CADHERIN AND ITS REPRESSOR SNAIL IN ENDOMETRIAL CANCER

4.1.1 Cancer tissue

In a first study, I have analyzed the active, i.e. nuclear localized, Snail protein and its target E-cadherin by immunohistochemistry in a series of primary tumors and metastases of estrogen-dependent EECs. It was of special interest to analyze Snail expression in this kind of cancer since Snail has been proposed to be repressed by Metastasis Tumor Antigen 3 (MTA3) expression. MTA3 in turn is supposed to be indirectly regulated through ERα signaling [41]. The results showed a significant association of reduced ERα expression in primary endometrial cancer with reduced E-cadherin immunoreactivity and positive Snail immunoreactivity, respectively. Reduced E-cadherin immunoreactivity and positive Snail immunoreactivity, respectively, were significantly correlated to a higher tumor grade in metastases of EECs. Positive Snail immunoreactivity was associated to reduced E-cadherin immunoreactivity only in metastatic lesions of endometrial cancer.

Reduced ERα expression was seen in 56.3% of the primary tumors, where it was significantly correlated to positive Snail ($p=0.047$) and to reduced E-cadherin immunoreactivity ($p=0.01$). 42.3% of the metastases showed reduced levels of ERα but no correlation was seen to Snail immunoreactivity and E-cadherin immunoreactivity. Nevertheless, a tendency for down-regulation or loss of ERα

expression was seen in Snail positive parts of the metastases, although it was statistically not significant. Reduced ERα immunoreactivity was found to be additionally correlated to a higher tumor grade in primary tumors but not in metastases of EECs. The lack of significance in the latter might be due to the smaller number of analyzed cases in the group of metastases. For the primary tumors these findings are in accordance to earlier reports which correlated estrogen receptor status to tumor stage and tumor grade [22, 75].

The role of E-cadherin in endometrial cancer has already been analyzed in previous studies where reduced E-cadherin expression was correlated to higher tumor grade [53, 123, 124], lower overall survival and tumor progression [69, 83]. In my study I found reduced E-cadherin expression in 44.8% of the primary tumors and in 65.4% of metastases of EECs. I observed a statistically significant correlation between E-cadherin down-regulation and higher tumor grade in the metastatic lesions (p=0.003) but not in the primary tumors. Abnormal E-cadherin expression was mainly seen in the more dedifferentiated parts of the tumors. Moreover, Holcomb *et al*. found that EECs were 23 times more likely to express E-cadherin than NEEC tumors when tumor grade was considered [53], and Scholten *et al*. reported negative E-cadherin expression to be significantly more often observed in NEECs than in EECs [127]. As mentioned above, reduced E-cadherin immunoreactivity was correlated to reduced ERα immunoreactivity in primary tumors of EECs but not in metastatic lesions. This finding maybe due to the smaller number of metastases analyzed. These results are in accordance to Fujita *et al*. [41] who showed an estrogen-dependent transcriptional regulation of E-cadherin through MTA3 expression which in turn represses Snail transcription in breast cancer cell lines.

Nothing is known so far about the expression of the E-cadherin repressor Snail in endometrial cancer. To my knowledge, this is the first study reporting immunohistochemical analysis of Snail in a series of endometrioid endometrial carcinomas. I found that 28.7% of the primary tumors and 53.8% of the metastases showed a positive nuclear staining for Snail. In addition to Snail positive carcinoma cells I also found stromal cells showing a positive immunoreactivity for Snail. Snail positive stromal cells have been described previously by Francí *et al*. [38] in colon cancer. This raises the question whether these Snail positive stromal cells represent former tumor cells that have undergone mesenchymal transition. On the other hand, a *de novo* expression of Snail in tumoral stroma might suggest a key role for stromal cells in promoting tumor progression. Several reports on breast cancer have provided evidence for the hypotheses that tumor stroma is cooperating in tumor progression and/or that these stromal cells might have been originated from the tumor [44, 84, 93, 105, 129]. Nevertheless, the role of Snail in tumor-associated stromal cells needs to be established in additional studies.

Snail immunoreactivity was significantly correlated to higher tumor grade in metastases (p=0.003) but not in primary tumors. I found positive Snail immunoreactivity mainly in the dedifferentiated solid parts of the primary tumors and rarely in the glandular areas. Taken together, these observations suggest that Snail may play a role in tumor dedifferentiation and progression. A statistically significant correlation between Snail up-regulation and E-cadherin down-regulation was also found in the metastases of EECs (p=0,003) but not in primary tumors. Snail expression did not lead to E-cadherin down-regulation in 56% of primary tumors. These results are in accordance with previous studies on colon cancer [38], although it is not yet understood why Snail expression does not lead to E-cadherin down-regulation in these cases. One hypothesis proposed by Francí *et al*. [38] is that Snail

and E-cadherin co-expressing cells are at the initial stages of the EMT process where the E-cadherin protein is still present, whereas Snail has already switched off E-cadherin gene expression. Another assumption is that Snail cofactors might be needed or a certain threshold must be reached for a successful down-regulation of E-cadherin. Recently, Peinado *et al*. [101] proposed a participation of different EMT regulators, e.g. Snail, Slug and SIP1, in E-cadherin down-regulation during tumor invasion, possibly complementing each other's action.

As mentioned above, positive Snail immunoreactivity was significantly correlated to reduced ERα expression in primary tumors but not in metastases of EECs. The latter might be due to the smaller number of cases analyzed in this group. The results gained by immunohistochemical analyses lead to the assumption that the reduction or loss of ERα signaling might be involved in the initial expression of Snail in primary tumors of the endometrium, but is not necessarily needed for the ongoing Snail expression. Nevertheless, reduced ERα expression was mainly seen in Snail positive parts of the metastatic lesions of EECs. As discussed above, Fujita *et al*. [41] already proposed a regulatory pathway through which Snail expression levels are indirectly influenced by ERα signaling. To address this question in more detail it might be of certain interest to compare Snail and ERα protein levels in the usually estrogen-related EECs to the hormone receptor negative NEECs.

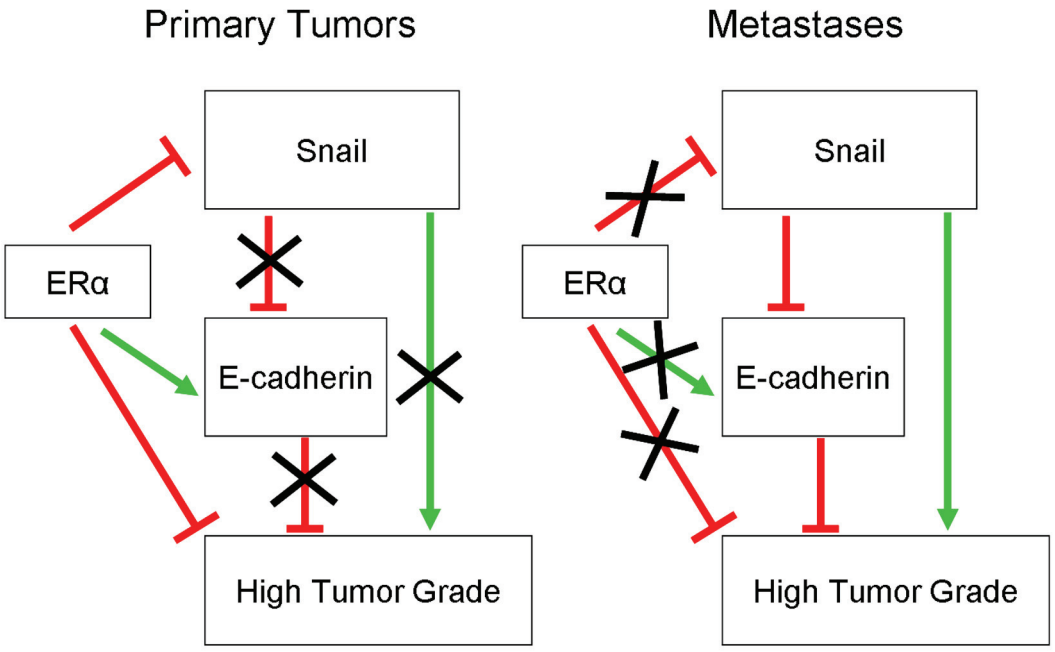

Figure 4-1: Schematic diagram of the regulatory mechanisms found in primary tumors and metastases of EECs. In primary tumors preserved ERα expression was correlated to preserved E-Cadherin expression, negative Snail expression and lower tumor grade. Neither E-cadherin nor Snail expression had an influence on the tumor grade. Snail expression was not correlated to reduced E-cadherin expression. In metastases ERα expression had no influence on the expression of E-cadherin or Snail nor on the tumor grade. Snail expression was correlated to a reduced E-cadherin expression and a higher tumor grade whereas preserved E-cadherin expression was associated with a lower tumor grade.

In addition to immunohistochemistry, I used Western Blot analysis for comparison of E-cadherin, Snail, and ERα protein expression in the same tissue areas in 11 formalin-fixed, paraffin-embedded primary human cancers, with 5 cases positive and 6 cases negative for ERα in immunohistochemistry. The technique of extracting high amounts of full length and immunoreactive proteins from formalin-fixed, paraffin-embedded tissues [8] enabled me to examine MTA3 in human tissues since the antibody used did not work for immunohistochemistry.

For ERα, the results obtained by Western Blot were concordant with the immunohistochemical findings. There was a single band of 67kDa for ERα in all 5 cases with positive ERα status in immunohistochemistry whereas the 6 cases with negative ERα status in immunohistochemistry showed no or in one case a very faint signal in the Western Blot experiments. Snail protein expression was seen in one ERα negative case but not in the ERα positive cases. Although these data have to be confirmed in a study with more tumor samples, this result underlines the immunohistochemical finding that Snail expression in primary tumors of EECs might be associated – at least in some cases – with reduced ERα expression. No significant differences were seen in the expression pattern of E-cadherin in ERα-negative compared to ERα-positive cases. Unexpectedly, I found expression of MTA3 in all 11 cases analyzed, independent of ERα status. Although my results have to be confirmed in a study comprising more endometrial cancer tissue samples, they are not concordant with the findings of Fujita *et al*. [42]. These authors investigated the molecular basis for estrogen and ER-dependent expression of MTA3 in breast cancer cell lines. They identified an estrogen response element (ERE) half-site in the MTA3 promoter and demonstrated loss of MTA3 transcript due to depletion of ERα by RNA interference.
In one case I observed a smaller variant of the MTA3 protein while expression of the expected form was reduced. The functional significance of this observation is yet not clear and has to be addressed in future experiments.

Taken together, these results (**Figure 4-1**) are in line with a proposed role for Snail in endometrial tumor progression, since Snail expression was correlated to higher tumor grade and reduced E-cadherin expression in metastases of EECs. Additionally, our results suggest that Snail expression may be in part influenced by the reduction or loss of ERα signaling. Nevertheless, it is questionable whether MTA3 is involved in this mechanism as MTA3 expression in the investigated tumor cases was independent of ERα status.

4.1.2 Endometrial cancer cell lines

In addition to the immunohistochemical analysis, I characterized four different endometrial carcinoma cell lines by Western Blot for the expression of E-cadherin, Snail, MTA3 and ERα. In three of the four endometrial carcinoma cell lines, namely Ishikawa, HEC-1-A and Ishikawa ER-, I found a correlation of low expression levels of Snail to high expression levels of E-cadherin and vice versa. This is in accordance to previous findings in other carcinoma cell lines [5, 64, 158], where Snail expression was shown to be inversely correlated to E-cadherin expression. The HEC-1-B endometrial carcinoma cell line showed neither E-cadherin expression nor high expression levels of Snail, suggesting that other repressor molecules, such as Slug, or another mechanism, e.g. protein destabilization, promoter methylation, is involved in E-cadherin down-regulation.

The results of the traditional wound-healing assay, which was performed to monitor cell motility, showed that the Snail-positive and E-cadherin-negative cell line Ishikawa ER- had a much higher migration potential than the Snail-negative and E-cadherin-positive cell line Ishikawa. These results are in agreement with earlier findings that Snail expression leads to the acquisition of invasive and migratory properties by down-regulating epithelial and up-regulating mesenchymal markers [21, 30]. Additional studies using siRNA knockdown of Snail in the Ishikawa ER- cells may be performed in the future to confirm the results.

Interestingly, only one of the two E-cadherin-positive cell lines showed ERα expression, whereas all four cell lines showed expression of MTA3 independent of the ERα expression status. This is again in contrast to the results of Fujita *et al.* [41], as mentioned above, who showed that in different breast cancer cell lines and in several breast tumor samples ERα signaling indirectly up-regulated the expression of MTA3, which in turn repressed the expression of Snail and therefore stabilized E-cadherin expression. Loss or down-regulation of ERα was associated with increased Snail expression and E-cadherin down-regulation. In a later study on breast cancer cells they linked MTA3 expression to ERα signaling by identifying a ERE half-site in the MTA3 promoter [42].

To further analyze this proposed regulation of Snail through ERα signaling and MTA3 expression in endometrial cancer cell lines I modulated ERα expression and signaling in the ERα-positive cell line Ishikawa.
A first attempt was knock-down of ERα expression by RNA interference (RNAi). RNAi has become widely used as an experimental tool to analyze the function of mammalian genes, both *in vitro* and *in vivo* [2]. Transfection of Ishikawa cells with ERα-specific siRNA led to a successful down-regulation of ERα expression as protein levels of ERα were markedly reduced on days 3 and 4 slowly re-increasing from day 5 to day 7 after siRNA transfection. However, the expression levels of Snail, MTA3, and E-cadherin remained totally unaffected by the altered ERα expression.
In a second attempt, I treated Ishikawa cells with the anti-estrogen fulvestrant. Fulvestrant is a 7α-alkylsulphinyl of 17β-estradiol belonging to a group of ligands called selective estrogen receptor modulators (SERMs). Unlike other non-steroidal members of this group, e.g. tamoxifen and raloxifene, fulvestrant is lacking partial agonistic activity [94]. Fulvestrant disrupts ER dimerisation and nuclear localization, completely blocking ER-mediated transcriptional activity and accelerating receptor degradation [23]. Indeed, ERα expression levels where markedly reduced in Ishikawa cells treated with fulvestrant over a time span of 6 days. No change in expression levels was seen for Snail, MTA3, and E-cadherin. Additionally, no change in the migration potential of the fulvestrant-treated Ishikawa cells was seen as monitored by a traditional wound-healing assay.
A third way to modulate ERα activity was a long time treatment of Ishikawa cells with 17β-estradiol (E_2). E_2 has been shown to lead to a rapid reduction in the steady-state levels of ER mRNA and protein in different breast cancer cell lines [66, 109]. This ligand-induced down-regulation of ER levels might contribute to the control of cell response to an estrogenic stimulus. To analyze this mechanism in endometrial carcinoma cells, Ishikawa cells were subjected to a long time treatment assay with E_2. During this treatment with E_2, ERα down-regulation in Ishikawa cells was observed which was ,surprisingly, accompanied by an up-regulation of cellular Snail protein levels. It is unlikely that the loss of ERα led to the induction of Snail expression as this was not the case when ERα was decreased due to RNA interference or treatment with the anti-estrogen fulvestrant. A possible explanation is

an effect of E_2 independent of ERα. Ding *et al.* described Snail mRNA up-regulation after E_2 treatment in the ERα-negative ovarian clear cell adenocarcinoma ES-2 cell line. They proposed a participation of ERβ or another, yet unknown, mechanism independent of the participation of ERα [31].

Taken together, the results of my cell culture studies suggest that MTA3 expression in the analyzed endometrial adenocarcinoma cell lines is independent of ERα expression status. Additionally, I could not confirm the proposed regulatory pathway through which Snail expression is supposed to be repressed by ERα signaling via MTA3 expression. Down-regulation of ERα expression in the endometrial cell line Ishikawa did not lead in two out of three examined cases to an up-regulation of Snail and did not affect MTA3 and E-cadherin expression, respectively, nor did it change the migratory properties of the cell line.

4.2 E-CADHERIN AND ITS REPRESSOR SNAIL IN OVARIAN CANCER

In a second study, I analyzed Snail and its target E-cadherin by immunohistochemistry in a series of primary ovarian carcinomas and corresponding metastases. The results show a significant association of reduced E-cadherin expression in primary ovarian cancer and positive Snail expression in corresponding metastases, respectively, with shorter overall survival of the patients. For both E-cadherin and Snail no significant differential expression between primary tumors and their corresponding metastases was observed.

E-cadherin has already been described to be involved in tumor dedifferentiation and poor recurrence free survival in ovarian cancer [58, 147]. In my study I found reduced E-cadherin immunoreactivity in 25% of primary ovarian tumors and in 22% of metastases. There was a significant correlation between E-cadherin expression in primary ovarian tumors and their corresponding metastases ($p<0.001$). Re-expression of E-cadherin at the metastatic site of the tumor has been observed in breast cancer metastases [18, 96] and metastases of ovarian carcinomas [58]. It is thought to enable tumor growth at the new site although the underlying mechanism is still unclear. These observations could not be confirmed for ovarian cancer in my study, as the majority (66.7%) of cases with reduced E-cadherin levels in the primary tumor also showed reduced E-cadherin immunoreactivity in the corresponding metastases. Nevertheless, 33.3% of cases with reduced E-cadherin immunoreactivity in primary tumors and 94.3% of cases with preserved E-cadherin immunoreactivity in primary tumors were E-cadherin positive in the corresponding metastases. This finding is in line with the hypothesis that E-cadherin is down-regulated during intermediate steps of metastasation, without being detected by immunohistochemical analysis, and re-expressed at the metastatic site.

No correlation was seen between reduced E-cadherin immunoreactivity and higher tumor grade or FIGO stage. This might be due to the fact that my study only included samples from patients with advanced ovarian cancer FIGO stages IIIC and IV, comprising 72.5% grade 3 tumors but only 21.6% grade 2 and 5.9% grade 1 tumors. No association was found between E-cadherin immunoreactivity and patient age or tumor subtype. However, I could show that reduced E-cadherin immunoreactivity in primary ovarian tumors was significantly associated with a lower overall survival of the patients ($p=0.008$). No association was found between E-cadherin expression in metastases of ovarian cancer and survival. These findings are in accordance to the

results of Faleiro-Rodrigues *et al*. [36] who analyzed 104 primary ovarian cancers for E-cadherin expression. They found no correlation between E-cadherin immunoreactivity and standard clinicopathological parameters, e.g. tumor grade, stage and patient age, but a significantly lower 5-year survival rate of patients with negative E-cadherin expression.

The expression of the transcription factor Snail has already been analyzed in previous studies. Nevertheless, these studies mainly focused on Snail mRNA expression and not on the subcellular localization of the protein. In a study which was published in 2005 Elloul *et al*. [34] evaluated the expression of E-cadherin, Snail, Slug and SIP1 mRNA in peritoneal and pleural effusions of 78 ovarian carcinomas and 23 breast carcinomas. Using RT-PCR they found Snail mRNA in 87% (68/78) ovarian carcinoma effusions and in 96% (22/23) breast carcinoma effusions, with significantly lower expression in ovarian carcinoma. In a more recent study, Elloul *et al*. [35] analyzed the expression of E-cadherin and its transcriptional regulators Snail, Slug and SIP1 in fresh, non-fixed material of ovarian primary carcinomas, solid metastases, and malignant peritoneal and pleural effusions by RT-PCR and Western Blot. The authors reported positive Snail expression in the majority of the analyzed tumor specimens. Snail mRNA expression was detected in 93% (38/41) of primary tumors, 93% (14/15) of metastases and 87% (68/78) of effusions. Snail protein expression determined by Western Blot analysis was found in 100% (30/30) of primary tumors, 100% (10/10) of metastases and 97% (72/74) of effusions. The authors found that mean expression levels of Snail protein were lower in effusions, with expression of 17% of control levels in effusions compared to 118% in primary tumors and 127% in metastases [35]. These discrepancies between Snail mRNA and protein expression levels may be explained by potential post-transcriptional and post-translational regulation mechanisms. Besides being tightly regulated at the transcriptional level, Snail´s activity is also influenced by its subcellular localization. In ovarian tumor cells derived from effusions, Elloul *et al*. found Snail protein to be exclusively localized in the cytoplasm [35], which may reflect an inactive form of the protein [32]. Snail can cycle between the nucleus and the cytosol by virtue of a nuclear export sequence (NES). At least two kinases, glycogen synthase kinase-3β (GSK3β) and p21-activated kinase 1 (PAK1), are known to govern Snail's localization [32, 157, 163]. In addition, the zinc-finger transporter LIV-1 seems to be involved in this level of regulation, as shown for zebrafish [156]. The complexity of Snail's functional activation makes clear that it is a shortcoming of Western Blot analysis that only total protein expression is reflected, which does not necessarily correlate to the amount of active Snail. Although not yet available, phospho-specific anti-Snail antibodies may be used in the future to determine the functional status of the EMT regulator.

In my study on ovarian cancer, I analyzed the expression of nuclear localized active Snail by immunohistochemistry. I found that 37.5% of the primary tumors and 52% of the corresponding metastases showed a positive nuclear staining for Snail. As for endometrial cancer, I also found tumor-associated stromal cells showing a positive immunoreactivity for Snail in addition to Snail positive carcinoma cells. As discussed above, expression of Snail in tumor-associated stromal cells might indicate a conversion of tumor cells into stromal cells by EMT and/or a possible role for these stromal cells in promoting tumor progression. However, the role of Snail in stromal cells is still unclear and needs further evaluation.

I found a significant correlation between Snail expression in primary ovarian tumors and their corresponding metastases ($p<0.001$). 94.1% of cases with a positive Snail

immunoreactivity in primary tumors were also Snail-positive in the corresponding metastases. On the other hand, 70% of cases with Snail-negative primary tumors were also Snail-negative in the corresponding metastases, yet 30% showed a positive Snail immunoreactivity. In previous studies, it was shown that Snail not only induced tumor invasion but also blocked the cell cycle and confers resistance to cell death [145]. My results indicate that these or other, yet unknown, features of Snail might be of special importance for the establishment or maintenance of metastases at the new invasion site in ovarian cancer possibly leading to preserved Snail expression. Additionally, I demonstrated that positive Snail immunoreactivity in metastases of ovarian cancer was significantly associated with a lower overall survival of the patients ($p=0.047$). No association was found between Snail expression in primary ovarian cancer and survival. Interestingly, in endometrial cancer I also observed the tumor progression promoting effects of Snail, i.e. E-cadherin down-regulation and correlation to a higher tumor grade, in the metastases of EECs but not in primary tumors. These findings might additionally underline a possible important role for Snail in metastatic lesions.

There was no correlation between Snail immunoreactivity and other clinicopathological parameters, including patient age, tumor subtype, or grade of differentiation. The last might be due to the small numbers of low-grade and low-stage cases as discussed above. Snail expression has already been correlated to tumor dedifferentiation in some cancers, e.g. in breast carcinomas [15]. For a better comparison regarding Snail expression and tumor grade and stage in ovarian cancer more cases comprising lower tumor grades and FIGO stages should be included in a future study.

In the analyzed ovarian cancer cases, there was no correlation between Snail up-regulation and E-cadherin down-regulation, neither in primary tumors nor in corresponding metastases. I found co-expression of E-cadherin and Snail in 36.1% of primary tumors and in 53.8% of metastases. These results are in accordance with my observations in primary tumors of EECs and with the findings of Francí *et al.* in colon cancer [38]. As discussed above, it is not clear why Snail and E-cadherin are co-expressed in these cases. It can only be speculated that this is due to Snail cofactors needed or because of a missing participation of other EMT regulators such as Slug or SIP1 as proposed by Peinado *et al*. [101].

I also asked whether specific combinations of E-cadherin and Snail protein expression had a prognostic value. I found that a profile of reduced E-cadherin expression and nuclear Snail expression was associated with a significantly increased risk of death. Patients showing an “E-cadherin-reduced and Snail-positive” profile, in either the primary tumors or corresponding metastases, respectively, had a 6-fold and 4.2-fold increased risk of death ($p=0.002$ and $p=0.022$, respectively) when compared to the patient group with a “normal epithelial” expression profile of “E-cadherin-positive and Snail-negative”. Additionally, patients with a profile of preserved E-cadherin and positive Snail expression in metastases were at an increased risk of death ($p=0.077$), although this association was not statistically significant.

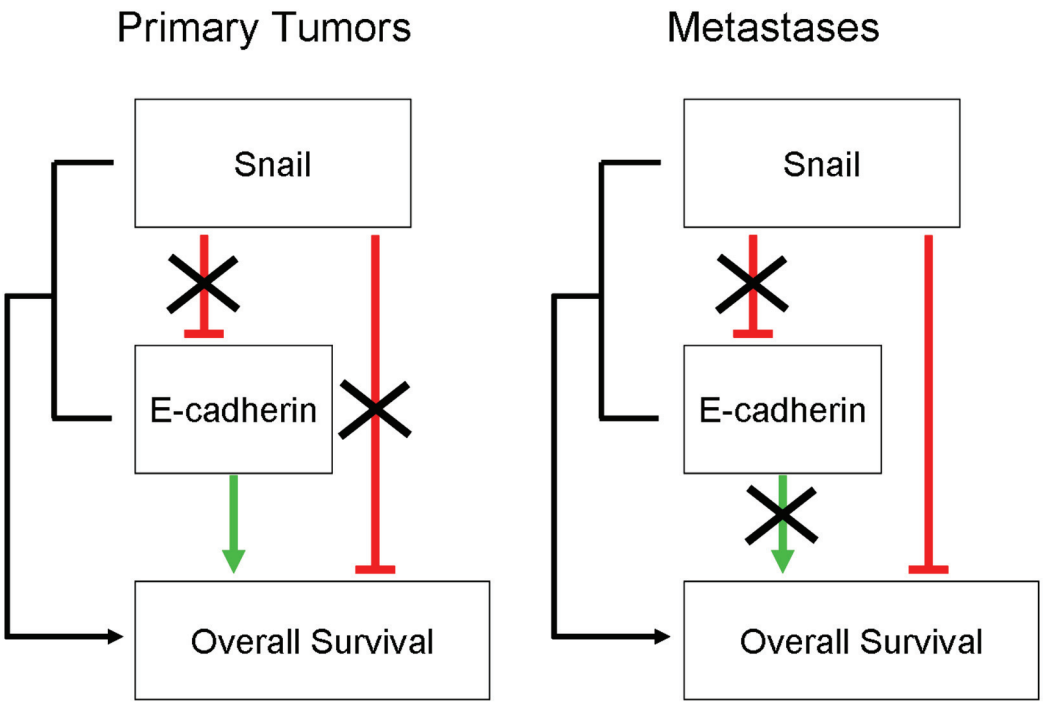

Figure 4-2: Schematic diagram of the influence of Snail and E-cadherin in primary tumors and metastases of ovarian carcinomas on the overall survival of patients. In primary tumors Snail expression had neither an influence on the expression of E-cadherin nor on the overall survival. However, preserved E-cadherin expression in primary tumors led to a significant longer overall survival of the patients. In metastases the expression of Snail was correlated to a significant shorter overall survival but not to reduced E-cadherin expression. In contrast to the primary tumors, the E-cadherin status in metastases had no effect on the survival of the patients. In both, primary tumors and metastases, patients showing the expression profile "E-cadherin-reduced and Snail-positive" had a significantly higher risk of death compared to patients with a "normal epithelial" expression profile of "E-cadherin-positive and Snail-negative".

A schematic diagram of the results of this study is shown in **Figure 4-2**. Taken together, these observations indicate that Snail might be an independent prognostic factor for clinical outcome in ovarian cancer. Snail may be important for the establishment and maintenance of metastases in ovarian cancer, while loss of E-cadherin expression might be crucial in primary tumors, e.g. for tumor invasion, both leading to an adverse clinical outcome for ovarian cancer patients.

4.3 E-CADHERIN AND ITS REPRESSOR SNAIL IN PLACENTAL TISSUE OF NORMAL, PREECLAMPTIC AND HELLP PREGNANCIES

It has often been said that the penetrative nature of human placentation mimics that of highly invasive tumors, the main difference being that there is a strict control in the extent of invasion. During the early stages of placental development trophoblastic cells adhere to, detach from, penetrate into, and finally adhere again to deeper

stromal tissues of the uterine wall. It has already been described that in course of the placental development these fetally-derived extravillous trophoblast (EVT) cells undergo epithelial to mesenchymal transition (EMT) [146]. This transition in human EVTs from the adherent to the invasive phenotype correlates with a change of cell surface adhesion molecule expression such as E-cadherin and integrins [29, 130, 165]. However, not much is known about Snail expression in EVTs. Especially its role in pathologic placental development, e.g. preeclampsia and HELLP syndrome, where the invasion potential of EVTs seems to be impaired, remains to be elucidated. To further analyze the role of Snail in placental development, I performed a third immunohistochemical study on tissue specimens of third trimester normal control, preeclamptic and HELLP syndrome placentas.

In the decidua of normal control placentas a strong E-cadherin immunoreactivity and a moderate Snail immunoreactivity was identified in EVTs. To prove that Snail- and E-cadherin-expressing cells in the decidual tissue are EVTs, I performed a fluorescence double staining with a cell marker (cytokeratin 7 for trophoblast cells, [77, 131]) and with either a Snail- or an E-cadherin-specific antibody. The staining with the monoclonal anti-E-cadherin antibody or the monoclonal anti-Snail antibody in combination with the anti-cytokeratin 7 antibody showed that the E-cadherin-/Snail-expressing cells were also positive for cytokeratin 7 and therefore of trophoblast origin. E-cadherin in third trimester placental villi (PV) anchoring to the superficial basal plate has already been described by Floridon *et al*. [37]. They showed that in the maternal–fetal junction zone E-cadherin-positive EVTs are found within areas of maternal cells and fibrinoid and were mainly grouped in large aggregates in the third trimester specimens [37]. In the maternal–fetal junction zone I found a significant reduction of E-cadherin immunoreactivity in preeclamptic EVTs compared to normal control placentas. A reduction of E-cadherin immunoreactivity was also observed in EVTs of HELLP placentas compared to normal control placentas although there was no statistical significance which might be due to the smaller case numbers analyzed in this group. Interestingly, in addition to the reduced E-cadherin immunoreactivity, I also identified a significant increase in Snail immunoreactivity in preeclamptic EVTs compared to the normal control. A similar increase in Snail immunoreactivity was seen in HELLP EVTs but without statistical significance. The immunohistochemical staining patterns of monoclonal antibodies against Snail and E-cadherin, respectively, are inversely correlated and therefore in good agreement with the above mentioned theory of a repression of E-cadherin by the transcription factor Snail. Nevertheless, this expression pattern is controversial to a speculation that the invasion of preeclamptic and HELLP syndrome EVTs may be limited due to an impaired EMT. In contrast to my results, Zhou *et al*. showed that in preeclamptic placental tissue cytotrophoblasts in both the villi and decidua showed strong reactivity with anti-E-cadherin, and staining remained strong even on cytotrophoblasts that had penetrated the superficial portions of uterine arterioles [164]. They claimed that a corresponding shallow interstitial and vascular invasion is the result in this condition [164]. Shallow invasion in preeclampsia is nonetheless a controversial finding [106]. The use of different anti-E-cadherin antibodies may at least in part explain the discrepancies in decidual staining.

On the other hand, I could also show that E-cadherin expression in the preeclamptic and HELLP villous trophoblast is elevated. For preeclamptic placental tissue this finding is in agreement with the results of Brown *et al*. [17] and Benian *et al*. [10]. These results suggest that enhanced immunoreactivity of E-cadherin in preeclamptic villous trophoblasts is accompanied by reduced immunoreactivity of this antigen in

the EVTs. Dysregulation of adhesion molecules that are involved in phenotypic changes associated with epithelial cell transformation are already known from other pathologic forms of pregnancy. Marzioni *et al*. [79] described down-regulation and/or dysregulation in expression of ZO-1 and occludin in normal human placenta and in hydatidiform moles. These molecules are also key molecules in cell-cell contacts like E-cadherin. In villous trophoblast cells a weak Snail immunoreactivity was observed in 1 out of 7 cases in normal placentas, 2 out of 7 cases in preeclamptic placentas and 2 out of 5 cases in HELLP placentas. Therefore it is difficult to speculate whether Snail is a key molecule for E-cadherin regulation in preeclamptic or HELLP villous trophoblasts.

Impaired invasion of uteroplacental arteries by EVTs is a key pathogenic mechanism of preeclampsia. Reister *et al.* previously demonstrated that reduced trophoblast invasion into uteroplacental spiral arteries was associated with an excess of macrophages in and around these arteries. Macrophages, residing in excess in the placental bed of preeclamptic women, are able to limit EVT invasion of spiral arterial segments through apoptosis mediated by the combination of TNFα secretion and tryptophan depletion [117]. However, the mechanisms by which macrophages are activated and recruited to the placental bed are presently unknown but are likely central to the pathogenesis of preeclampsia. Jeschke *et al.* reported a significantly reduced expression of glycodelin protein in decidual cells of preeclamptic and HELLP syndrome placentas, respectively [63]. Glycodelin is known to possess immunosuppressive properties, i.e. inhibition of natural killer (NK) cell [91] and T cell [112] activity as well as induction of T cell apoptosis [111]. Reduced expression of glycodelin in the maternal-fetal interface might therefore be accompanied by an increased activation of the maternal immune response.

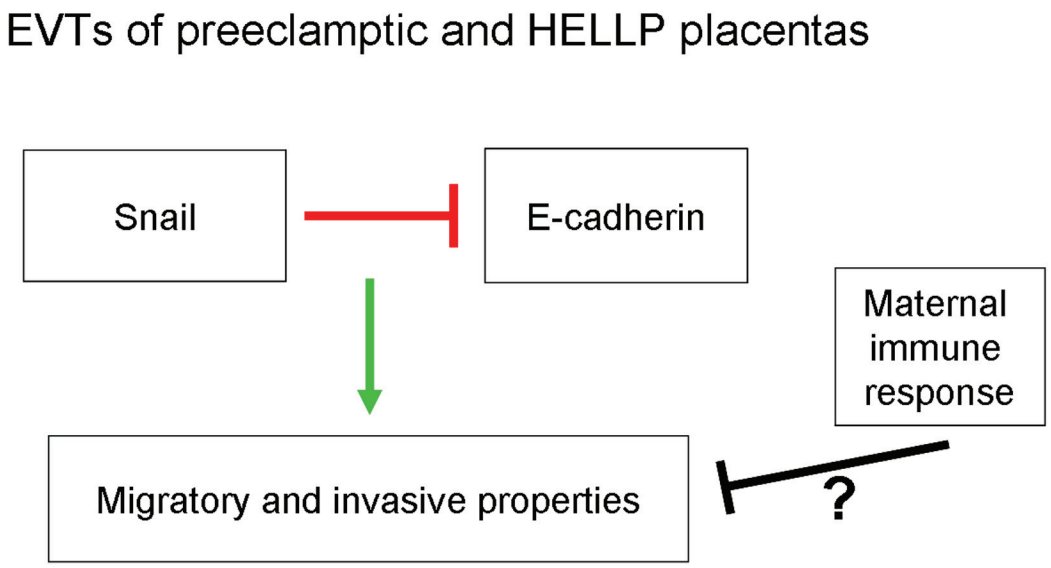

Figure 4-3: Schematic diagram of the proposed regulatory mechanisms found in EVTs of preeclamptic and HELLP syndrome placentas. Up-regulation of Snail expression in preeclamptic and HELLP EVTs was accompanied by reduced E-Cadherin expression in the same cells suggesting a direct regulation of E-Cadherin by Snail in EVTs. The migratory and invasive properties of preeclamptic and HELLP EVTs might therefore be reduced by an altered maternal immune response independent of EMT.

The data obtained in my study show that in preeclamptic and HELLP syndrome EVTs Snail is up-regulated and E-cadherin is down-regulated. This expression scheme suggests that migration of EVTs is not disturbed in preeclampsia and HELLP syndrome (**Figure 4-3**). Invasion may be limited, independent of EMT, by an altered maternal immune response, e.g. by activated macrophages in preeclamptic placentas and induction of apoptosis in EVTs. Further investigations on migration of preeclamptic EVTs are necessary to proof these speculations.

4.4 CONCLUSIONS

During my work I have performed the first studies in which the subcellular expression of the E-cadherin repressor Snail has been analyzed in a series of EECs, ovarian carcinomas and normal and pathologic placental tissue.

In conclusion, the results of my studies show that the transcription factor Snail is differentially expressed in primary tumors and metastases of EECs and provide new evidences for a role of Snail in tumor progression of endometrioid adenocarcinomas. The findings concerning a participation of ERα signaling in Snail regulation are controversial. The results in cancer tissue specimens indicate a possible role for ERα in Snail regulation – at least in primary tumors – whereas the cell culture experiments do not confirm these findings. In both, endometrial cancer tissue and cell lines, MTA3 could neither be shown to be regulated by ERα signaling nor to influence Snail expression levels.
In ovarian cancer, Snail is associated with lower overall survival of the patients. The results indicate that Snail plays a role as a prognostic factor for adverse clinical outcome in this kind of cancer.
In a physiological model for migration and invasion, i.e. placental development during embryo implantation, I detected Snail expression in normal and pathologic EVTs. Up-regulation of Snail expression in preeclamptic and HELLP syndrome EVTs is accompanied by reduced E-cadherin expression in the same cells suggesting a direct regulation of E-cadherin by Snail in EVTs. These results suggest that the invasion potential of preeclamptic and HELLP syndrome EVTs might be reduced by other mechanisms, e.g. maternal immune responses, independent of EMT.

5 SUMMARY

This work elucidates the role of the epithelial-mesenchymal transition (EMT) regulator Snail in tumors of the feminine genital tract and reports the first studies in which the active, i.e. nuclear localized, Snail protein has been analyzed in a great variety of gynaecological patient tissue specimens. In addition, functional analyses were performed in four cell lines.

In a first study on endometrial cancer I analyzed 113 tissue samples of primary tumors and metastatic lesions of endometrioid adenocarcinomas. By focussing on this type of estrogen-related endometrial cancer, I intended to gain further insight into the role of Snail in cancer progression and elucidate a proposed regulatory mechanism of Snail via estrogen receptor α (ERα) signaling. 28.7% of primary tumor specimens and 53.8% of the metastases showed a positive Snail immunoreactivity. This was correlated to reduced ERα immunoreactivity ($p=0.047$) in primary tumors and to both higher tumor grade ($p=0.003$) and abnormal E-cadherin expression ($p=0.003$) in metastatic lesions. Taken together, these data are in line with a proposed role for Snail in endometrial tumor progression. In addition, I analyzed four different endometrial adenocarcinoma cell lines. A Snail-expressing cell line showed a higher migration potential than a variant of this cell line with low levels of Snail. Down-regulation of ERα in an ERα- and E-cadherin-positive and Snail-negative endometrial adenocarcinoma cell line did not result in up-regulation of Snail or down-regulation of E-cadherin. A long-time stimulation with 17β-estradiol did result in up-regulation of Snail.

In a second study on ovarian cancer, I analyzed 98 tissues of primary ovarian carcinomas and their corresponding metastases. I found a significant correlation of E-cadherin expression in primary cancers ($p=0,008$) and Snail expression in corresponding metastases ($p=0.047$), respectively, with overall survival of the patients. Additionally, patients showing positive Snail and reduced E-cadherin expression in primary tumors and corresponding metastases, respectively, had a significantly higher risk for the occurrence of death ($p=0.002$ and $p=0.022$, respectively) when compared to the reference group with the normal epithelial expression profile, i.e. E-cadherin-positive and Snail-negative.

A physiological model for migration and invasion which shows many similarities to the growth of cancer cells, is placental development during embryo implantation. Therefore, in addition to tumor tissues, I included specimens of normal placenta and placenta from pregnancies complicated with preeclampsia and HELLP syndrome. These complications in pregnancy are thought to be associated with incomplete invasion of extravillous trophoblasts (EVTs). In preeclamptic and HELLP EVTs Snail immunoreactivity was increased, accompanied by a reduction of E-cadherin immunoreactivity. These results suggest that the invasion potential of preeclamptic and HELLP syndrome EVTs might be reduced by other mechanisms, e.g. maternal immune responses, independent of EMT.

Taken together, the data of my studies underline a role for Snail in tumor progression and adverse clinical outcome for patients with gynaecological cancers, although regulation of Snail through ERα signaling, as proposed by others, could not be confirmed *in vitro*, and indicate a possible involvement of Snail in placental development during embryo implantation.

6 REFERENCES

1. **Akhurst RJ, Balmain A.** (1999) Genetic events and the role of TGF beta in epithelial tumour progression. *J Pathol* **187:**82-90.

2. **Alexander HK, Booy EP, Xiao W, Ezzati P, Baust H, Los M.** (2007) Selected technologies to control genes and their products for experimental and clinical purposes. *Arch Immunol Ther Exp (Warsz)* **55:**139-149.

3. **Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I.** (2005) Endometrial cancer. *Lancet* **366:**491-505.

4. **Babawale MO, Van Noorden S, Pignatelli M, Stamp GW, Elder MG, Sullivan MH.** (1996) Morphological interactions of human first trimester placental villi co-cultured with decidual explants. *Hum Reprod* **11:**444-450.

5. **Batlle E, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A.** (2000) The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* **2:**84-89.

6. **Beato M.** (1989) Gene regulation by steroid hormones. *Cell* **56:**335-344.

7. **Becker KF, Rosivatz E, Blechschmidt K, Kremmer E, Sarbia M, Höfler H.** (2007) Analysis of the E-cadherin repressor Snail in primary human cancers. *Cells Tissues Organs* **185:**204-212.

8. **Becker KF, Schott C, Hipp S, Metzger V, Porschewski P, Beck R, Nahrig J, Becker I, Hofler H.** (2007) Quantitative protein analysis from formalin-fixed tissues: implications for translational clinical research and nanoscale molecular diagnosis. *J Pathol* **211:**370-378.

9. **Behrens J, Lowrick O, Klein-Hitpass L, Birchmeier W.** (1991) The E-cadherin promoter: functional analysis of a G.C-rich region and an epithelial cell-specific palindromic regulatory element. *Proc Natl Acad Sci U S A* **88:**11495-11499.

10. **Benian A, Madazli R, Aksu F, Uzun H, Aydin S.** (2002) Plasma and placental levels of interleukin-10, transforming growth factor-beta1, and epithelial-cadherin in preeclampsia. *Obstet Gynecol* **100:**327-331.

11. **Bernards R.** (2003) Cancer: cues for migration. *Nature* **425:**247-248.

12. **Berx G, Becker KF, Hofler H, van Roy F.** (1998) Mutations of the human E-cadherin (CDH1) gene. *Hum Mutat* **12:**226-237.

13. **Berx G, Staes K, van Hengel J, Molemans F, Bussemakers MJ, van Bokhoven A, van Roy F.** (1995) Cloning and characterization of the human invasion suppressor gene E-cadherin (CDH1). . *Genomics* **26:**281-289.

14. **Birchmeier W, Behrens J.** (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* **1198:**11-26.

15. **Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, Nieto MA.** (2002) Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene* **21:**3241-3246.

16. **Bokhman JV.** (1983) Two pathogenetic types of endometrial carcinoma. *Gynecol Oncol* **15:**10-17.

17. **Brown LM, Lacey HA, Baker PN, Crocker IP.** (2005) E-cadherin in the assessment of aberrant placental cytotrophoblast turnover in pregnancies complicated by pre-eclampsia. *Histochem Cell Biol* **124:**499-506.

18. **Bukholm IK, Nesland JM, Borresen-Dale AL.** (2000) Re-expression of E-cadherin, alpha-catenin and beta-catenin, but not of gamma-catenin, in metastatic tissue from breast cancer patients. *J Pathol* **190:**15-19.

19. **Bussemakers MJ, Giroldi LA, van Bokhoven A, Schalken JA.** (1994) Transcriptional regulation of the human E-cadherin gene in human prostate cancer cell lines: characterization of the human E-cadherin gene promoter. *Biochem Biophys Res Commun* **203:**1284-1290.

20. **Cannistra SA.** (2004) Cancer of the ovary. *N Engl J Med* **351:**2519-2529.

21. **Cano A, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA.** (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* **2:**76-83.

22. **Carcangiu ML, Chambers JT, Voynick IM, Pirro M, Schwartz PE.** (1990) Immunohistochemical evaluation of estrogen and progesterone receptor content in 183 patients with endometrial carcinoma. Part I: Clinical and histologic correlations. *Am J Clin Pathol* **94:**247-254.

23. **Carlson RW.** (2005) The history and mechanism of action of fulvestrant. *Clin Breast Cancer* **6 Suppl 1:**S5-8.

24. **Carver EA, Jiang R, Lan Y, Oram KF, Gridley T.** (2001) The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* **21:**8184-8188.

25. **Cavallaro U, Christofori G.** (2001) Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. *Biochim Biophys Acta* **1552:**39-45.

26. **Comijn J, Berx G, Vermassen P, Verschueren K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F.** (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* **7:**1267-1278.

27. **Correa P, Shiao YH.** (1994) Phenotypic and genotypic events in gastric carcinogenesis. *Cancer Res* **54:**1941s-1943s.

28. **Cross JC, Werb Z, Fisher SJ.** (1994) Implantation and the placenta: key pieces of the development puzzle. *Science* **266:**1508-1518.

29. **Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, Zhou Y, Logan SK, Fisher SJ.** (1994) Integrin switching regulates normal trophoblast invasion. *Development* **120:**3657-3666.

30. **De Craene B, Gilbert B, Stove C, Bruyneel E, van Roy F, Berx G.** (2005) The transcription factor snail induces tumor cell invasion through modulation of the epithelial cell differentiation program. *Cancer Res* **65:**6237-6244.

31. **Ding JX, Feng YJ, Yao LQ, Yu M, Jin HY, Yin LH.** (2006) The reinforcement of invasion in epithelial ovarian cancer cells by 17beta-Estradiol is associated with up-regulation of Snail. *Gynecol Oncol*.

32. **Dominguez D, Montserrat-Sentis B, Virgos-Soler A, Guaita S, Grueso J, Porta M, Puig I, Baulida J, Franci C, Garcia de Herreros A.** (2003) Phosphorylation regulates the subcellular location and activity of the snail transcriptional repressor. *Mol Cell Biol* **23:**5078-5089.

33. **Duckitt K, Harrington D.** (2005) Risk factors for pre-eclampsia at antenatal booking: systematic review of controlled studies. *Bmj* **330:**565.

34. **Elloul S, Elstrand MB, Nesland JM, Trope CG, Kvalheim G, Goldberg I, Reich R, Davidson B.** (2005) Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. *Cancer* **103:**1631-1643.

35. **Elloul S, Silins I, Trope CG, Benshushan A, Davidson B, Reich R.** (2006) Expression of E-cadherin transcriptional regulators in ovarian carcinoma. *Virchows Arch* **449:**520-528.

36. **Faleiro-Rodrigues C, Macedo-Pinto I, Pereira D, Lopes CS.** (2004) Prognostic value of E-cadherin immunoexpression in patients with primary ovarian carcinomas. *Ann Oncol* **15:**1535-1542.

37. **Floridon C, Nielsen O, Holund B, Sunde L, Westergaard JG, Thomsen SG, Teisner B.** (2000) Localization of E-cadherin in villous, extravillous and vascular trophoblasts during intrauterine, ectopic and molar pregnancy. *Mol Hum Reprod* **6:**943-950.

38. **Franci C, Takkunen M, Dave N, Alameda F, Gomez S, Rodriguez R, Escriva M, Montserrat-Sentis B, Baro T, Garrido M, Bonilla F, Virtanen I, Garcia de Herreros A.** (2006) Expression of Snail protein in tumor-stroma interface. *Oncogene* **25:**5134-5144.

39. **Franci C, Takkunen M, Dave N, Alameda F, Gomez S, Rodriguez R, Escriva M, Montserrat-Sentis B, Baro T, Garrido M, Bonilla F, Virtanen I, Garcia de Herreros A.** (2006) Expression of Snail protein in tumor-stroma interface. *Oncogene*.

40. **Fujimoto J, Ichigo S, Hirose R, Sakaguchi H, Tamaya T.** (1997) Expression of E-cadherin and alpha- and beta-catenin mRNAs in uterine cervical cancers. *Tumour Biol* **18:**206-212.

41. **Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA.** (2003) MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. *Cell* **113:**207-219.

42. **Fujita N, Kajita M, Taysavang P, Wade PA.** (2004) Hormonal regulation of metastasis-associated protein 3 transcription in breast cancer cells. *Mol Endocrinol* **18:**2937-2949.

43. **Fujita Y, Krause G, Scheffner M, Zechner D, Leddy HE, Behrens J, Sommer T, Birchmeier W.** (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* **4:**222-231.

44. **Galie M, Sorrentino C, Montani M, Micossi L, Di Carlo E, D'Antuono T, Calderan L, Marzola P, Benati D, Merigo F, Orlando F, Smorlesi A, Marchini C, Amici A, Sbarbati A.** (2005) Mammary carcinoma provides highly tumourigenic and invasive reactive stromal cells. *Carcinogenesis* **26:**1868-1878.

45. **Gotzmann J, Mikula M, Eger A, Schulte-Hermann R, Foisner R, Beug H, Mikulits W.** (2004) Molecular aspects of epithelial cell plasticity: implications for local tumor invasion and metastasis. *Mutat Res* **566:**9-20.

46. **Grau Y, Carteret C, Simpson P.** (1984) Mutations and chromosomal rearrangements affecting the expression of Snail, a gene involved in embryonic patterning in Drosophila Melanogaster. *Genetics* **108:**347-360.

47. **Grille SJ, Bellacosa A, Upson J, Klein-Szanto AJ, van Roy F, Lee-Kwon W, Donowitz M, Tsichlis PN, Larue L.** (2003) The protein kinase Akt induces epithelial mesenchymal transition and promotes enhanced motility and invasiveness os squamous cell carcinoma lines. *Cancer Res* **63:**2172-2178.

48. **Guaita S, Puig I, Franci C, Garrido M, Dominguez D, Batlle E, Sancho E, Dedhar S, de Herreros AG.** (2002) Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. *J Biol Chem* **277:**39209-39216.

49. **Hay ED.** (1995) An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* **154:**8-20.

50. **Hay ED.** (2005) The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Dev Dyn* **233:**706-720.

51. **Hemavathy K, Ashraf SI, Ip YT.** (2000) Snail/slug family of repressors: slowly going into the fast lane of development and cancer. *Gene* **257:**1-12.

52. **Hirohashi S.** (1998) Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* **153:**333-339.

53. **Holcomb K, Delatorre R, Pedemonte B, McLeod C, Anderson L, Chambers J.** (2002) E-cadherin expression in endometrioid, papillary serous, and clear cell carcinoma of the endometrium. *Obstet Gynecol* **100:**1290-1295.

54. **Hsu MY, Meier FE, Nesbit M, Hsu JY, Van Belle P, Elder DE, Herlyn M.** (2000) E-cadherin expression in melanoma cells restores keratinocyte-mediated growth control and down-regulates expression of invasion-related adhesion receptors. *Am J Pathol* **156:**1515-1525.

55. **Huiping C, Kristjansdottir S, Jonasson JG, Magnusson J, Egilsson V, Ingvarsson S.** (2001) Alterations of E-cadherin and beta-catenin in gastric cancer. *BMC Cancer* **1:**16.

56. **Humphrey GW, Wang Y, Russanova VR, Hirai T, Qin J, Nakatani Y, Howard BH.** (2001) Stable histone deacetylase complexes distinguished by the presence of SANT domain proteins CoREST/kiaa0071 and Mta-L1. *J Biol Chem* **276:**6817-6824.

57. **Ikenouchi J, Matsuda M, Furuse M, Tsukita S.** (2003) Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci* **116:**1959-1967.

58. **Imai T, Horiuchi A, Shiozawa T, Osada R, Kikuchi N, Ohira S, Oka K, Konishi I.** (2004) Elevated expression of E-cadherin and alpha-, beta-, and gamma-catenins in metastatic lesions compared with primary epithelial ovarian carcinomas. *Hum Pathol* **35:**1469-1476.

59. **Imai T, Horiuchi A, Wang C, Oka K, Ohira S, Nikaido T, Konishi I.** (2003) Hypoxia attenuates the expression of E-cadherin via up-regulation of SNAIL in ovarian carcinoma cells. *Am J Pathol* **163:**1437-1447.

60. **Ishida N, Hara T, Kamura T, Yoshida M, Nakayama K, Nakayama KI.** (2002) Phosphorylation of p27kip1 on serine 10 is required for ist binding to CRM1 and nuclear export. *J Biol Chem* **277:**14355-14358.

61. **Jawhari A, Jordan S, Poole S, Browne P, Pignatelli M, Farthing MJ.** (1997) Abnormal immunoreactivity of the E-cadherin-catenin complex in gastric carcinoma: relationship with patient survival. *Gastroenterology* **112:**46-54.

62. **Jeffers MD, Paxton J, Bolger B, Richmond JA, Kennedy JH, McNicol AM.** (1997) E-cadherin and integrin cell adhesion molecule expression in invasive and in situ carcinoma of the cervix. *Gynecol Oncol* **64:**481-486.

63. **Jeschke U, Kunert-Keil C, Mylonas I, Hammer A, Schiessl B, Lomba I, Kuhn C, Schulze S, Friese K.** (2005) Expression of glycodelin A in decidual

tissue of preeclamptic, HELLP and intrauterine growth-restricted pregnancies. *Virchows Arch* **446:**360-368.

64. **Jiao W, Miyazaki K, Kitajima Y.** (2002) Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo. *Br J Cancer* **86:**98-101.

65. **Jones SL.** (1998) HELLP! A cry for laboratory assistance: a comprehensive review of the HELLP syndrome highlighting the role of the laboratory. *Hematopathol Mol Hematol* **11:**147-171.

66. **Journe F, Body JJ, Leclercq G, Nonclercq D, Laurent G.** (2004) Estrogen responsiveness of IBEP-2, a new human cell line derived from breast carcinoma. *Breast Cancer Res Treat* **86:**39-53.

67. **Kadar N, Homesley HD, Malfetano JH.** (1994) Prognostic factors in surgical stage III and IV carcinoma of the endometrium. *Obstet Gynecol* **84:**983-986.

68. **Kadar N, Malfetano JH, Homesley HD.** (1992) Determinants of survival of surgically staged patients with endometrial carcinoma histologically confined to the uterus: implications for therapy. *Obstet Gynecol* **80:**655-659.

69. **Kim YT, Choi EK, Kim JW, Kim DK, Kim SH, Yang WI.** (2002) Expression of E-cadherin and alpha-, beta-, gamma-catenin proteins in endometrial carcinoma. *Yonsei Med J* **43:**701-711.

70. **Kingdom J, Huppertz B, Seaward G, Kaufmann P.** (2000) Development of the placental villous tree and its consequences for fetal growth. *Eur J Obstet Gynecol Reprod Biol* **92:**35-43.

71. **Ko H, Kim HS, Kim NH, Lee SH, Kim KH, Hong SH, Yook JI.** (2007) Nuclear localization signals of the E-cadherin transcriptional repressor Snail. *Cells Tissues Organs* **185:**66-72.

72. **LaBonne C, Bronner-Fraser M.** (2000) Snail-related trandcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev Biol* **221:**195-205.

73. **Lala PK, Hamilton GS.** (1996) Growth factors, proteases and protease inhibitors in the maternal-fetal dialogue. *Placenta* **17:**545-555.

74. **Lapidus RG, Nass SJ, Davidson NE.** (1998) The loss of estrogen and progesterone receptor gene expression in human breast cancer. *J Mammary Gland Biol Neoplasia* **3:**85-94.

75. **Lax SF.** (2004) Molecular genetic pathways in various types of endometrial carcinoma: from a phenotypical to a molecular-based classification. *Virchows Arch* **444:**213-223.

76. **Leblanc M, Poncelet C, Soriano D, Walker-Combrouze F, Madelenat P, Scoazec JY, Darai E.** (2001) Alteration of CD44 and cadherins expression:

possible association with augmented aggressiveness and invasiveness of endometrial carcinoma. *Virchows Arch* **438:**78-85.

77. **Manyonda IT, Whitley GS, Cartwright JE.** (2001) Trophoblast cell lines: a response to the Workshop Report by King et al. *Placenta* **22:**262-263.

78. **Marques FR, Fonsechi-Carvasan GA, De Angelo Andrade LA, Bottcher-Luiz F.** (2004) Immunohistochemical patterns for alpha- and beta-catenin, E- and N-cadherin expression in ovarian epithelial tumors. *Gynecol Oncol* **94:**16-24.

79. **Marzioni D, Banita M, Felici A, Paradinas FJ, Newlands E, De Nictolis M, Muhlhauser J, Castellucci M.** (2001) Expression of ZO-1 and occludin in normal human placenta and in hydatidiform moles. *Mol Hum Reprod* **7:**279-285.

80. **Matias-Guiu X, Catasus L, Bussaglia E, Lagarda H, Garcia A, Pons C, Munoz J, Arguelles R, Machin P, Prat J.** (2001) Molecular pathology of endometrial hyperplasia and carcinoma. *Hum Pathol* **32:**569-577.

81. **Mattaj IW, Englmeier L.** (1998) Nucleocytoplasmic transport: the soluble phase. *Annu Rev Biochem* **67:**265-306.

82. **Mauhin V, Lutz Y, Dennefeld C, Alberga A.** (1993) Definition of the DNA-binding site repertoire for the Drosophila transcription factor SNAIL. *Nucleic Acids Res* **21:**3951-3957.

83. **Mell LK, Meyer JJ, Tretiakova M, Khramtsov A, Gong C, Yamada SD, Montag AG, Mundt AJ.** (2004) Prognostic significance of E-cadherin protein expression in pathological stage I-III endometrial cancer. *Clin Cancer Res* **10:**5546-5553.

84. **Moinfar F, Man YG, Arnould L, Bratthauer GL, Ratschek M, Tavassoli FA.** (2000) Concurrent and independent genetic alterations in the stromal and epithelial cells of mammary carcinoma: implications for tumorigenesis. *Cancer Res* **60:**2562-2566.

85. **Moll R, Mitze M, Frixen UH, Birchmeier W.** (1993) Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am J Pathol* **143:**1731-1742.

86. **Morrow CP, Bundy BN, Kurman RJ, Creasman WT, Heller P, Homesley HD, Graham JE.** (1991) Relationship between surgical-pathological risk factors and outcome in clinical stage I and II carcinoma of the endometrium: a Gynecologic Oncology Group study. *Gynecol Oncol* **40:**55-65.

87. **Myatt L, Miodovnik M.** (1999) Prediction of preeclampsia. *Semin Perinatol* **23:**45-57.

88. **Nieto MA.** (2002) The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* **3:**155-166.

89. **Nollet F, Kools P, Van Roy F.** (2000) Phylogenetic analysis of the cadherin superfamily allows identification of six major superfamilies besides several solitary members. *J Mol Biol* **299:**551-572.

90. **Ohkubo T, Ozawa M.** (2004) The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation. *J Cell Sci* **117:**1675-1685.

91. **Okamoto N, Uchida A, Takakura K, Kariya Y, Kanzaki H, Riittinen L, Koistinen R, Seppala M, Mori T.** (1991) Suppression by human placental protein 14 of natural killer cell activity. *Am J Reprod Immunol* **26:**137-142.

92. **Okamura H, Aramburu J, Garcia-Rodriguez C, Viola JPB, Raghavan A, Tahiliani M, Zhang X, Qin J, Hogan PG, Rao A.** (2000) Concerted dephosphorylation of the transcription factor NFAT1 induces a conformational switch that regulates transcriptional activity. *Mol Cell* **6:**539-550.

93. **Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA.** (2005) Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell* **121:**335-348.

94. **Osborne CK, Wakeling A, Nicholson RI.** (2004) Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer* **90 Suppl 1:**S2-6.

95. **Ozols RF.** (2005) Treatment goals in ovarian cancer. *Int J Gynecol Cancer* **15 Suppl 1:**3-11.

96. **Park D, Karesen R, Axcrona U, Noren T, Sauer T.** (2007) Expression pattern of adhesion molecules (E-cadherin, alpha-, beta-, gamma-catenin and claudin-7), their influence on survival in primary breast carcinoma, and their corresponding axillary lymph node metastasis. *Apmis* **115:**52-65.

97. **Parkin DM, Bray F, Ferlay J, Pisani P.** (2005) Global cancer statistics, 2002. *CA Cancer J Clin* **55:**74-108.

98. **Pearce ST, Jordan VC.** (2004) The biological role of estrogen receptors alpha and beta in cancer. *Crit Rev Oncol Hematol* **50:**3-22.

99. **Peinado H, Ballestar E, Esteller M, Cano A.** (2004) Snail mediates E-cadherin repression by the recruitment of the Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex. *Mol Cell Biol* **24:**306-319.

100. **Peinado H, Olmeda D, Cano A.** (2007) Snail, ZEB and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* **7:**415-428.

101. **Peinado H, Portillo F, Cano A.** (2004) Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* **48:**365-375.

102. **Peinado H, Quintanilla M, Cano A.** (2003) Transforming growth factor beta 1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem* **278:**21113-21123.

103. **Perez-Moreno M, Jamora C, Fuchs E.** (2003) Sticky business: orchestrating cellular signals at adherens junctions. *Cell* **112:**535-548.

104. **Perez-Moreno M, Locascio A, Rodrigo I, Dhondt G, Portillo F, Nieto MA, Cano A.** (2001) A new role for E12/E47 in the repression of E-cadherin expression and epithelial-mesenchymal transitions. *J Biol Chem* **276:**27424-27431.

105. **Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Rank F, Niebuhr E, Bissell MJ, Ronnov-Jessen L.** (2003) Epithelial to mesenchymal transition in human breast cancer can provide a nonmalignant stroma. *Am J Pathol* **162:**391-402.

106. **Pijnenborg R.** (1998) The origin and future of placental bed research. *Eur J Obstet Gynecol Reprod Biol* **81:**185-190.

107. **Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L, van Assche A.** (1991) Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Br J Obstet Gynaecol* **98:**648-655.

108. **Pijnenborg R, Dixon G, Robertson WB, Brosens I.** (1980) Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta* **1:**3-19.

109. **Pink JJ, Jordan VC.** (1996) Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. *Cancer Res* **56:**2321-2330.

110. **Poser I, Dominguez D, de Herreros AG, Varnai A, Buettner R, Bosserhoff AK.** (2001) Loss of E-cadherin expression in melanoma cells involves up-regulation of the transcriptional repressor Snail. *J Biol Chem* **276:**24661-24666.

111. **Rachmilewitz J, Riely GJ, Huang JH, Chen A, Tykocinski ML.** (2001) A rheostatic mechanism for T-cell inhibition based on elevation of activation thresholds. *Blood* **98:**3727-3732.

112. **Rachmilewitz J, Riely GJ, Tykocinski ML.** (1999) Placental protein 14 functions as a direct T-cell inhibitor. *Cell Immunol* **191:**26-33.

113. **Ranscht B.** (1994) Cadherins and catenins: interactions and functions in embryonic development. *Curr Opin Cell Biol* **6:**740-746.

114. **Rashid MG, Sanda MG, Vallorosi CJ, Rios-Doria J, Rubin MA, Day ML.** (2001) Posttranslational truncation and inactivation of human E-cadherin

distinguishes prostate cancer from matched normal prostate. *Cancer Res* **61:**489-492.

115. **Rath W, Bartz C.** (2004) [Treatment of severe preeclampsia and HELLP syndrome]. *Zentralbl Gynakol* **126:**293-298.

116. **Rath W, Faridi A, Dudenhausen JW.** (2000) HELLP syndrome. *J Perinat Med* **28:**249-260.

117. **Reister F, Frank HG, Kingdom JC, Heyl W, Kaufmann P, Rath W, Huppertz B.** (2001) Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. *Lab Invest* **81:**1143-1152.

118. **Remmele W, Stegner HE.** (1987) [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. *Pathologe* **8:**138-140.

119. **Rodrigo I, Cato AC, Cano A.** (1999) Regulation of E-cadherin gene expression during tumor progression: the role of a new Ets-binding site and the E-pal element. *Exp Cell Res* **248:**358-371.

120. **Rosivatz E, Becker I, Specht K, Fricke E, Luber B, Busch R, Hofler H, Becker KF.** (2002) Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* **161:**1881-1891.

121. **Rosivatz E, Becker KF, Kremmer E, Schott C, Blechschmidt K, Hofler H, Sarbia M.** (2006) Expression and nuclear localization of Snail, an E-cadherin repressor, in adenocarcinomas of the upper gastrointestinal tract. *Virchows Arch* **448:**277-287.

122. **Ryan AJ, Susil B, Jobling TW, Oehler MK.** (2005) Endometrial cancer. *Cell Tissue Res* **322:**53-61.

123. **Saito T, Nishimura M, Yamasaki H, Kudo R.** (2003) Hypermethylation in promoter region of E-cadherin gene is associated with tumor dedifferention and myometrial invasion in endometrial carcinoma. *Cancer* **97:**1002-1009.

124. **Sakuragi N, Nishiya M, Ikeda K, Ohkouch T, Furth EE, Hareyama H, Satoh C, Fujimoto S.** (1994) Decreased E-cadherin expression in endometrial carcinoma is associated with tumor dedifferentiation and deep myometrial invasion. *Gynecol Oncol* **53:**183-189.

125. **Sankaranarayanan R, Ferlay J.** (2005) Worldwide burden of gynaecological cancer: The size of the problem. *Best Pract Res Clin Obstet Gynaecol* **20:**207-225.

126. **Schlessinger K, Hall A.** (2004) GSK-3beta sets Snail's pace. *Nat Cell Biol* **6:**913-915.

127. **Scholten AN, Aliredjo R, Creutzberg CL, Smit VT.** (2006) Combined E-cadherin, alpha-catenin, and beta-catenin expression is a favorable prognostic factor in endometrial carcinoma. *Int J Gynecol Cancer* **16:**1379-1385.

128. **Shames BD, Fernandez LA, Sollinger HW, Chin LT, D'Alessandro AM, Knechtle SJ, Lucey MR, Hafez R, Musat AI, Kalayoglu M.** (2005) Liver transplantation for HELLP syndrome. *Liver Transpl* **11:**224-228.

129. **Shekhar MP, Werdell J, Santner SJ, Pauley RJ, Tait L.** (2001) Breast stroma plays a dominant regulatory role in breast epithelial growth and differentiation: implications for tumor development and progression. *Cancer Res* **61:**1320-1326.

130. **Shih Ie M, Hsu MY, Oldt RJ, 3rd, Herlyn M, Gearhart JD, Kurman RJ.** (2002) The Role of E-cadherin in the Motility and Invasion of Implantation Site Intermediate Trophoblast. *Placenta* **23:**706-715.

131. **Shiverick KT, King A, Frank H, Whitley GS, Cartwright JE, Schneider H.** (2001) Cell culture models of human trophoblast II: trophoblast cell lines--a workshop report. *Placenta* **22 Suppl A:**S104-106.

132. **Sibai BM.** (2004) Diagnosis, controversies, and management of the syndrome of hemolysis, elevated liver enzymes, and low platelet count. *Obstet Gynecol* **103:**981-991.

133. **Sibai BM, Caritis S, Hauth J.** (2003) What we have learned about preeclampsia. *Semin Perinatol* **27:**239-246.

134. **Sobin LH, Wittekind C.** (2002) TNM Classification of Malignant Tumours. Wiley-Liss, New York, p. 240.

135. **Strathdee G.** (2002) Epigenetic versus genetic alterations in the inactivation of E-cadherin. *Semin Cancer Biol* **12:**373-379.

136. **Takeichi M.** (1990) Cadherins: a molecular family important in selective cell-cell adhesion. *Annu Rev Biochem* **59:**237-252.

137. **Takeichi M.** (1991) Cadherin cell adhesion receptors as a morphogenetic regulator. *Science* **251:**1451-1455.

138. **Tavassoli FA, Devilee P.** (2003) World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs. IARC Press, Lyon, p. 432.

139. **Thiery JP.** (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2:**442-454.

140. **Thiery JP.** (2003) Epithelial-mesenchymal transitions in development and pathologies. *Curr Opin Cell Biol* **15:**740-746.

141. **Thiery JP, Chopin D.** (1999) Epithelial cell plasticity in development and tumor progression. *Cancer Metastasis Rev* **18:**31-42.

142. **Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL.** (1998) Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. *Nature* **395:**917-921.

143. **Twigg SR, O. WA.** (1999) Characterisation of the human snail (SNAI1) gene and exclusion as a major disease gene in craniosynostosis. *Hum Genet* **105:**320-326.

144. **Vandromme M, Gauthier-Rouviere C, Lamb N, Fernandez A.** (1996) Regulation of transcription factor localization: fine-tuning of gene expression. *Trends Biochem Sci* **21:**59-64.

145. **Vega S, Morales AV, Ocana OH, Valdes F, Fabregat I, Nieto MA.** (2004) Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev* **18:**1131-1143.

146. **Vicovac L, Aplin JD.** (1996) Epithelial-mesenchymal transition during trophoblast differentiation. *Acta Anat (Basel)* **156:**202-216.

147. **Voutilainen KA, Anttila MA, Sillanpaa SM, Ropponen KM, Saarikoski SV, Juhola MT, Kosma VM.** (2006) Prognostic significance of E-cadherin-catenin complex in epithelial ovarian cancer. *J Clin Pathol* **59:**460-467.

148. **Wade PA, Gegonne A, Jones PL, Ballestar E, Aubry F, Wolffe AP.** (1999) Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. *Nat Genet* **23:**62-66.

149. **Wade PA, Jones PL, Vermaak D, Wolffe AP.** (1998) A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. . *Curr Biol* **8:**843-846.

150. **Walker JJ.** (2000) Pre-eclampsia. *Lancet* **356:**1260-1265.

151. **Weinstein L.** (1982) Syndrome of hemolysis, elevated liver enzymes, and low platelet count: a severe consequence of hypertension in pregnancy. *Am J Obstet Gynecol* **142:**159-167.

152. **Wheeler JM, Kim HC, Efstathiou JA, Ilyas M, Mortensen NJ, Bodmer WF.** (2001) Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. *Gut* **48:**367-371.

153. **Whittemore AS.** (1994) Characteristics relating to ovarian cancer risk: implications for prevention and detection. *Gynecol Oncol* **55:**S15-19.

154. **Xue Y, Wong J, Moreno GT, Young MK, Cote J, Wang W.** (1998) NURD, a novel complex with both ATPase-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* **2:**851-861.

155. **Yamasaki H, Sekimoto T, Ohkubo T, Douchi T, Nagata Y, Ozawa M, Yoneda Y.** (2005) Zinc finger domain of Snail functions as anuclear localization signal for importin beta-mediated nuclear import pathway. *Genes Cells* **10:**455-464.

156. **Yamashita S, Miyagi C, Fukada T, Kagara N, Che YS, Hirano T.** (2004) Zinc transporter LIVI controls epithelial-mesenchymal transition in zebrafish gastrula organizer. *Nature* **429:**298-302.

157. **Yang Z, Rayala S, Nguyen D, Vadlamudi RK, Chen S, Kumar R.** (2005) Pak1 phosphorylation of snail, a master regulator of epithelial-to-mesenchyme transition, modulates snail's subcellular localization and functions. *Cancer Res* **65:**3179-3184.

158. **Yokoyama K, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R, Nagayama M.** (2001) Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. *Oral Oncol* **37:**65-71.

159. **Yoneda Y, Hieda M, Nagoshi E, Miyamoto Y.** (1999) Nucleocytoplasmic protein transport and recycling of Ran. *Cell Struct Funct* **24:**425-433.

160. **Yuan Y, Hilliard G, Ferguson T, Millhorn DE.** (2003) Cobalt inhibits the interaction between hypoxia-inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia-inducible factor-alpha. *J Biol Chem* **278:**15911-15916.

161. **Zhang Y, LeRoy G, Seelig HP, Lane WS, Reinberg D.** (1998) The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities. *Cell* **95:**279-289.

162. **Zhang Y, Xiong Y.** (2001) A p53 amino-terminal nuclear export signal inhibited by DNA damage-induced phosphorylation. *Science* **292:**1910-1915.

163. **Zhou BP, Deng J, Xia W, Xu J, Li YM, Gunduz M, Hung MC.** (2004) Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* **6:**931-940.

164. **Zhou Y, Damsky CH, Fisher SJ.** (1997) Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *J Clin Invest* **99:**2152-2164.

165. **Zhou Y, Fisher SJ, Janatpour M, Genbacev O, Dejana E, Wheelock M, Damsky CH.** (1997) Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J Clin Invest* **99:**2139-2151.

7 ABBREVIATIONS

aa amino acid
aqua dest. (*aqua destillata*) distilled water
BSA bovine serum albumin
CI confidence interval
Cy2 carbocyanin
Cy3 indocarbocyanin
DAB 3,3'-diaminobenzidine
DMEM Dulbecco's Modified Eagle's Medium
DMSO dimethylsulfoxide
E_2 17β-estradiol
EDTA ethylenediaminetetraacetic acid
EEC endometrioid carcinoma of the endometrium
E-cad. E-cadherin
EMT epithelial-mesenchymal transition
ER estrogen receptor
ERE estrogen response element
$EtOH_{absolute}$ ethanol (purity $\geq$ 99.9%)
EVT extravillous trophoblast
FCS fetal calf serum
FFPE formalin-fixed, paraffin-embedded
FIGO International Federation of Gynecology and Obstetrics
GSK-3β glycogen synthase kinase-3β
HELLP hemolysis, elevated liver enzymes, low platelets
HR hazard ratio
HRP horseradish peroxidase
IHC immunohistochemistry
IRS immunoreactivity score
MTA3 metastasis associated protein 3
NEEC non-endometrioid carcinoma of the endometrium
neg. CTRL negative control
NES nuclear export sequence
PAGE polyacrylamid gel electrophoresis
Pak1 p21-activated kinase 1
PBS phosphate buffered saline
PCR polymerase chain reaction
PE preeclampsia
PVDF polyvinylidenfluorid
RNAi RNA interference
RT-PCR reverse transcriptase polymerase chain reaction
SEM standard error of the mean
SERM selective estrogen receptor modulator
SIP1 Smad-interacting protein 1
siRNA short interfering RNA
TEMED N,N,N',N'-tetramethylethylene diamine
TNF tumor necrosis factor
UICC International Union Against Cancer
WHO World Health Organization

8 ANNOTATION

Parts of this work have been published in:

„Virchows Archiv“ (Blechschmidt, K., Mylonas, I., Mayr, D., Schiessl, B., Schulze, S., Becker, K.-F., Jeschke, U (2007) Expression of E-cadherin and its repressor Snail in placental tissue of normal, preeclamptic and HELLP pregnancies. Virchows Arch. 450:195-202)

„Diagnostic Molecular Pathology“ (Blechschmidt, K., Kremmer, E., Hollweck, R., Mylonas, I., Höfler, H., Kremer, M., Becker, K.-F. (2007) The E-cadherin repressor Snail plays a role in tumor progression of endometrioid adenocarcinomas. Diagn Mol Pathol. 16:222-228)

„British Journal of Cancer“ (Blechschmidt, K., Sassen, S., Schmalfeldt, B., Schuster, T., Höfler, H., Becker, K.-F. (2008) The E-cadherin repressor Snail is associated with lower overall survival of ovarian cancer patients. Br J Cancer 98:489-495)