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INDUCTION OF EPITHELIAL-MESENCHYMAL TRANSITION (EMT) IN WOMEN´S CANCER: PROTECTIVE ROLE OF DIFFERENTIATION FACTORS

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Induction of Epithelial-Mesenchymal Transition (EMT) in Women´s cancer: Protective Role of Differentiation Factors

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“The most difficult thing is to know what we do know, and what we do not know”

Tertium Organum, 1920, P. D. Ouspensky

ABSTRACT

Metastatic spread of cancer cells to vital organs is the predominant cause of death among women suffering from breast and ovarian cancer, and invasive cancer cells are in many cases resilient to standard drugs used in the clinic. Consequently, further understanding of the metastatic process and development of new strategies to target invasive cancer cells are needed. One process that has been closely linked to cancer cell invasion and migration is epithelial-mesenchymal transition (EMT), a developmental process, which can be reactivated during cancer progression. EMT allows carcinoma cells, with an epithelial origin, to acquire mesenchymal and migratory properties that are employed to invade the surrounding tumor tissue. The overall aim of this thesis was to investigate how EMT is induced in breast and ovarian cancer cells and to study the role of EMT in drug resistance.

Relapse of resilient cancer cells after surgery and first line of drug treatments is a major cause of death in ovarian and breast cancer. Currently, little is known about the functional properties of cancer cells that develop resistance to existing drug treatments and how they can be targeted. The aim of **study I** was to characterize the phenotypic properties of ovarian cancer cells that developed resistance to cisplatin, a chemotherapeutic drug commonly used in the clinic. We found that human SKOV-3 ovarian cancer cells that acquired resistance to cisplatin gained properties of EMT and cancer stem cells, suggesting that they were more invasive than drug-sensitive cells. Indeed, functional experiments showed that cisplatin-resistant SKOV-3 cells were more migratory in invasion assays and displayed an increased tumor initiating capacity compared to cisplatin-sensitive cells. The results from these studies link EMT to drug resistance in ovarian cancer cells, and emphasize that further understanding of EMT is needed and to be able to target EMT for therapy.

In **study II-IV** we investigated how cellular sensitivity to EMT is regulated. In particular, we focused on identifiying epithelial differentiation factors that regulate EMT in breast cancer cells. We identified two transcription factors – C/EBPβ and Foxp4 that were lost during breast cancer progression, which conferred cells an enhanced capacity to undergo EMT as well as to gain invasive and metastatic properties in experimental *in vitro* and *in vivo* models of breast cancer. In addition, we identified the coxsackie- and adenovirus receptor (CAR), a tight junction-based cell adhesion molecule, as a novel regulator of Akt signaling and TGF-β-induced EMT in breast cancer cells. The mechanism was traced to a role of CAR in regulating localization, stability and function of the phosphatase Pten, a potent Akt inhibitor, at tight junctions. The results from these studies indicate that the EMT process is not solely regulated by factors that drive a mesenchymal differentiation program, but also, is under tight control by epithelial differentiation factors. Loss of C/EBPβ, Foxp4 and CAR may lead to increased cellular sensitivity to EMT and thereby open up the possibility that cancer cells acquire invasive and migratory properties. Based on this, we propose that novel therapies aiming to strengthen, or preserve, epithelial differentiation mechanisms in breast or ovarian cancer cells, might be useful as a type of differentiation therapy to inhibit cancer cell invasion and metastasis.

LIST OF SCIENTIFIC PAPERS

Included in the thesis:

I. My Wintzell, Lina Löfstedt, **Joel Johansson**, Pedersen AB, Jonas Fuxe, and Maria Shoshan. Repeated cisplatin treatment can lead to a multiresistant tumor cell population with stem cell features and sensitivity to 3-bromopyruvate. Cancer Biol Ther., 2012 Dec 1;13(14):1454-62

II. Joel Johansson *, Tove Berg*,Ewa Kurzejamska , Meifong Pang, Vedrana Tabor, Malin Jansson, Pernilla Roswall, Kristian Pietras, Malin Sund, Piotr Religa and Jonas Fuxe, MiR-155-mediated loss of C/EBPβ shifts the TGF-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer, Oncogene 2013, Aug 19. doi: 10.1038/onc.2013.322

III. Joel Johansson, Magdalena Cichon, Laura Lambut, Vedrana Tabor, Anna Wikell, Malin Sund, Derek Radisky, Jonas Fuxe. Foxp4 controls EMT in breast cancer cells by acting as a transcriptional repressor of Snail1, *Manuscript*

IV. Joel Johansson, Sandra Travica, Azadeh Nilchian, Vedrana Tabor, Oskar Rosenkrantz, Malin Sund, Theresa Vincent, Jonas Fuxe. The Coxsackie- and Adenovirus Receptor Controls Akt Signaling and EMT in Breast Cancer Cells by Regulating Pten Localization and Stability at Tight Junctions, *Manuscript*

Others not included in the thesis:

V. Joel Johansson*, Vedrana Tabor*, Anna Wikell, Sirpa Jalkanen and Jonas Fuxe, TGF-β1-induced epithelial-mesenchymal transition induces monocyte/macrophage properties in breast cancer cells, Submitted manuscript (in press), Frontiers in Oncology, 2014

VI. Ewa Kurzejamska, **Joel Johansson**, Karin Jirström, Varsha Prakash, Sharan Ananthaseshan, Louis Boon, Jonas Fuxe, Piotr Religa, C/EBPβ expression is an independent predictor of overall survival in breast cancer patients by MHCII/CD4-dependent mechanism of metastasis formation. Oncogenesis, 2014 Nov 3;3:e125. doi: 10.1038/oncsis.2014.38.

VII. Mei-Fong Pang, Anna-Maria Georgoudaki, Laura Lambut, **Joel Johansson**, Vedrana Tabor, Kazuhiro Hagikura, Yi Jin, Malin Jansson, Lars Jakobsson, Christer Betsholtz, Malin Sund, Mikael C. I. Karlsson and Jonas Fuxe, TGF-b1-induced EMT promotes dendritic cell properties and lymphatic dissemination of breast cancer cells, Submitted manuscript (in revision). Oncogene, 2014

**equal contribution*

CONTENTS

LIST OF ABBREVIATIONS

Akt/PkB	Protein kinase B
AP-1	Activating Protein 1
CAR	Coxsackie- and adenovirus receptor
C/EBPb	CCAAT-enhancer binding protein beta
CLMP	CXADR-like membrane protein
CSC	Cancer Stem Cell
EGF	Epidermal Growth Factor
EMT	Epithelial-Mesenchymal Transition
EOC	Epithelial Ovarian cCncer
Foxp4	Forkhead box p4
GSK-3β	Glykogensyntas-kinas 3 beta
HER2	Human Epidermal Growth Factor Receptor 2
HK-II	Hexokinase-II
JAM	Junctional Adhesion Molecule
MAGI-1	Membrane Associated Guanylate Kinase, WW and PDZ Domain Containing 1
MMP	Matrix Metalloproteinases
MET	Mesenchymal-Epithelial Transition
NMuMG	Namru Mouse MammaryGland
PyMT	Polyma Virus Middle-T
PR	Progesterone Receptor
Pten	Phosphatase and Tensin Homolog
PI3K	Phosphatidylinositol 3-kinase
RHOA	Ras homolog gene family, member A
RAC1	Rho family, small GTP binding protein
RAS	Rat Sarcoma Protein
SP-1	Specificity Protein 1
TAK1	TGFβ activated kinase
TJ	Tight Junction
TGFβ	Transforming Growth Factor Beta
TGFβR	Transforming Growth Factor Beta Recptor

I INTRODUCTION

1.1 Women´s Cancer

Development of the human embryo, from one initial germ cell to a functional multi-cellular organism, is dependent on predetermined events of cellular transitions. In a controlled fashion, stem cells divide and differentiate into a number of different cell types, with the capacity to build up and maintain the physical functions of our body. This is made possible when cells follow proper genetic programs, which coordinate the course of development and the cellular response to external conditions.[1,2] Cancer cells no longer follow proper genetic programs, and lose their ability to act in accordance with needs of the multi-cellular organism. In later stage of the cancerous disease, the potent force of transition is once more triggered and tumor cells not only divide rapidly, but change their morphology and turn invasive.[3,4,5]

Women´s cancer comprises of malignancies of the cervical, ovarian, and breast epithelia and in more rare cases the vaginal and vulvar epithelia.[6] The incident of death of cervical cancer patients has largely been reduced in several countries due to successful screening programs. In addition, vaccine against oncogenic human papilloma virus is likely to further reduce the incidence and death caused by cervical cancer.[7] However for breast cancer, the most common malignant disease among women in the western world, and ovarian cancer breakthroughs in finding new therapeutic alternatives are well needed.[8,9] In particular, it would be of interest to identify and be able to target the mechanism that drives metastatic spread of breast and ovarian cancer cells.

1.2 Breast cancer progression

Breast tumor progression cannot be linked to mutations in a single pathway, and is rather a heterogeneous disease with different pathologies and molecular profiles.[10,11] Initially, human breast tumors were categorized based on their histological features, but more recently gene expression profiling based on microarray analysis has made it possible to classify human breast tumors based on to their mRNA expression profile.[12,13] Through mRNA profiles it is possible to divide tumors into four reproducible subtypes: luminal A, luminal B, basal and normal-like.[12,13,14] Most subtypes of breast cancer are luminal tumors that share morphology with the inner epithelial cells facing the lumen of the mammary ducts. Luminal A tumors are positive for estrogen and the progesterone receptor (ER and PR) and negative for HER2, which makes them responsive to hormone treatment, and have a better prognosis.[15,16] Luminal B tumors are positive for HER2, and the prognosis is poorer due to increased cell growth.[17] Basal like tumors do not share the luminal morphology but instead resemble myoepithelial (or basal) cells lining the basement membrane of the mammary duct.[18] Tumors with this type of morphology are more invasive and migratory.[19]

Usually it is not the primary breast tumor that is the cause of death in patients, but rather metastatic spread to distant sites.[20] Although the metastatic process is far from understood, advances with in the field have enabled us to grasp some of its complexity. Once tumor cells surpass the anti-proliferative control of tumor suppressor genes, such as p53, the retinoblastoma protein (RB) and Phosphatase and tensin homolog (Pten), and provide their own growth stimulatory factors they start to divide in an uncontrolled fashion.[3,21,22,23,24,25] The general dogma and the explanation model of how tumors progress into an invasive state has

been that fast dividing tumor cells will accumulate more mutations that eventually allow them to acquire invasive properties. The balance between the activity of oncogenes, which may promote an invasive behavior, and tumor suppressors may play a role in dictating the invasive behavior of tumor cells.[26,27,28] This still holds valid, but accumulating data point towards higher levels of complexity, and that intrinsic events cannot solely explain why certain breast cancer cells acquire invasive properties. Although tumor cells have lost their normal proliferative behavior, they are still growing in a context where many components come together to determine the course of progression (Figure 1).[3,29] It is evident that both extracellular signals and intracellular events are important for the invasive behavior of tumor cells.

Mammary ducts are encapsulated by both a basement membrane and the surrounding stromal compartment containing extracellular matrix. Thus, no preexisting natural passages exists that allow mammary epithelial cells to migrate through in order to reach the blood circulation.[30] The basement membrane is an insoluble structure that is impermeable to large proteins, and the focal extracellular matrix is only permeable to cell movement during tissue remodeling, wound healing, inflammation and neoplasia. [31,32,33,34] A first step of progression is therefore for tumor cells to attach to the basement membrane which is mediated by specific glycoproteins such as laminins and fibronectin, through plasma membrane receptors.[32,35,36] When attached, tumor cells secret hydrolytic enzymes that break up the basement membrane and start to degrade the matrix. [37,38,39]

As tumor cells pass the boarder of what usually separates the epithelial and mesenchymal compartment they are exposed to a new microenvironment that has the potential to further modify the tumor cells.[40,41,42] The interaction with the tumor microenvironment is not a one way communication, and during tumor progression cancer cells also influence its own environment. This is evident, in the way tumor cells recruit bone marrow derived cells such as leukocytes, neutrophils and macrophages to the tumor stroma, that in turn interact with tumor cells e.g. by producing cytokines.[43,44,45] Under these conditions, when both the intrinsic factors of the tumor cells and the pro metastatic microenvironment act together, tumor cells can turn motile and invasive.[38,46]

As tumor cells turn invasive, they have the capacity to migrate towards blood and lymphatic vessels and intravasate into the circulation.[47] Tumor cells are known to produce pro angiogenic factors such as VEGF-A and VEGF-C, which promote both angiogenesis and lymphangiogenesis respectively.[48,49,50] In the tumor micro environment blood and lymphatic vessels will in turn produce factors stimulating tumor cells, e.g. lymphatic vessels are known to produce chemokines that attract migratory tumor cells [51]. The blood vessels that are formed in the tumor microenvironment are known to be leaky and often lack normal coverage by pericytes and vascular smooth muscle cells, which makes them permeable, and tumor cells can disseminate to the circulation.[52,53] Lymph vessels are on the other hand designed for trafficking of immune cells, and can also act as a route tumor cells into the circulation.[54,55,56] Once in the circulation, breast cancer cells have the capacity to find and to form metastasis in distant organs such as lung, bone and liver.[57,58]

1.3 Epithelial Ovarian Cancer Progression

Although a new classification of ovarian cancer, based on genetic profiles, has been developed dividing ovarian cancer into two broad groups of type I and type II, ovarian cancers are still divided into different subtypes including serious, endometrioid, clear cell, mucinous, and undifferentiated tumors based on morphological features. Serious carcionomas

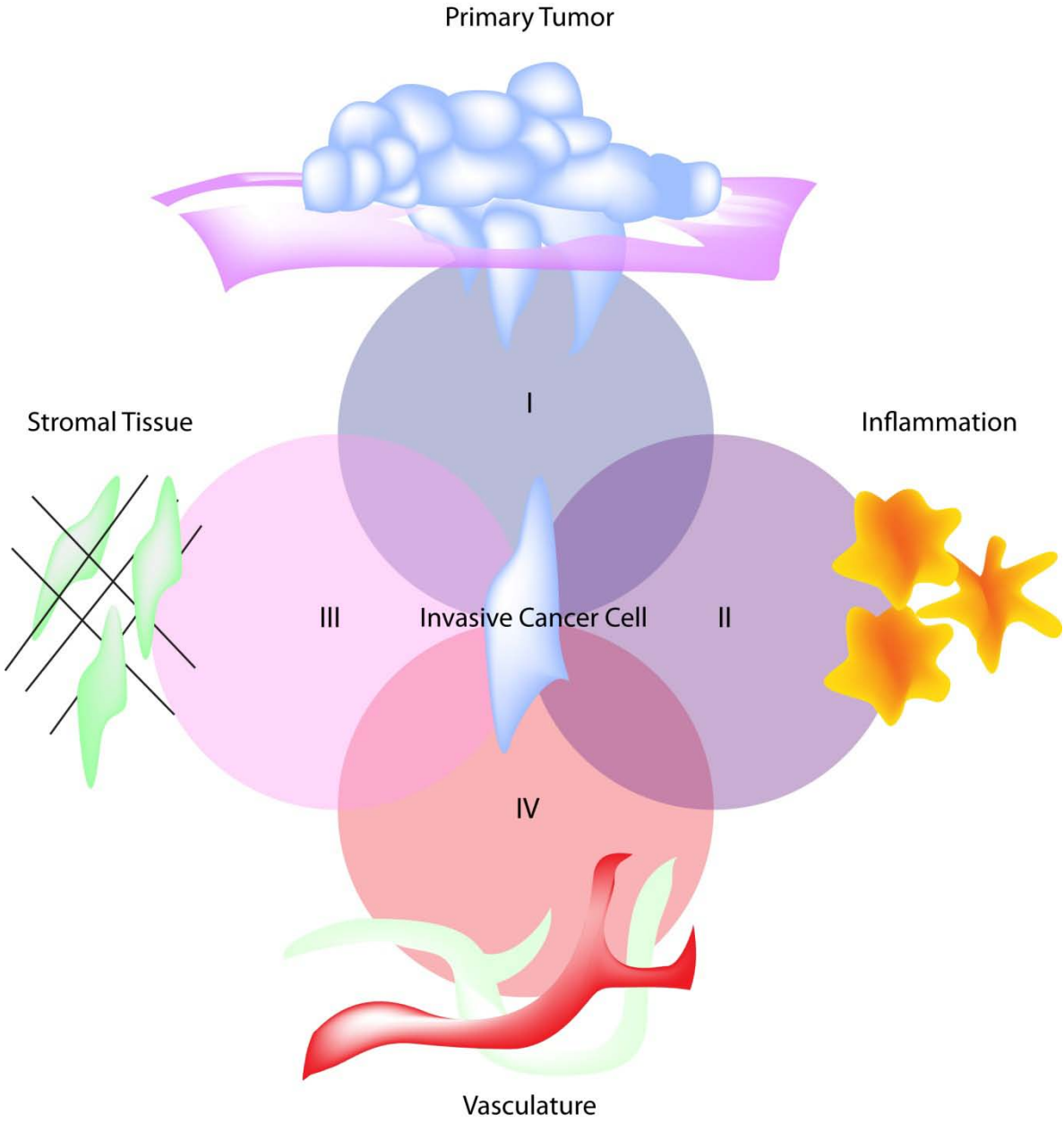

Figure 1. Crosstalk between components in the tumor microenvironment and tumor cells drive tumor progression and invasion. Initially tumors grow in a confined epithelial compartment which is separated from underlying mesenchymal tissues by the basement membrane of the mammary duct. Throughout progression tumor cells secrete several factors such as EGF which establish (I) a paracrine circuit between tumor cells to sustain growth, and later as tumor cells enter the mesenchyme involve (II) recruitment of inflammatory cells with the capacity to either suppress or promote tumor progression, and (III) remodulation of the tumor stroma and interaction with stromal cells. Finally, tumor cells (IV) produce angiogenic factors that affect surrounding vasculature resulting in angiogenesis. In turn vessels produce chemokines serving as a gradient for tumor cells to migrate to. As tumor cells establish these connections, the route leading to blood circulation, becomes available.

are the most common types tumors of all EOC, and are divided into low (type I group) and high (type II group) grades tumors.[6,59]

Different from many other types of cancers, ovarian cancer has generally not been linked to chemical carcinogens with mutagenic potential, and only a few mutations of e.g. p53, typical for serious high grade carcinomas, has been linked to onset of ovarian cancers.[60,61,62] Epithelial cells of the ovary are known to proliferate at a low rate and generally stay quiescent. Only after ruptures of mature follicles to release oocytes, eptheilial cells have to proliferate in order to repair the disrupted ovarian surface.[63] A large fraction of ovarian cancer develops at the epithelial subsurface of inclusion cysts, formed during ovulation.[64]

Factors that increase the number of ovulatory cycles, such as early onset of menses and late menopause also increase the risk of acquiring ovarian cancer. Opposite, a reduced number of ovulatory cycles decrease the risk, and these factors include e.g. multiple pregnancies and prolonged lactation. It is difficult to detect early stages (I/II) of ovarian cancer since there are few specific symptoms of the disease and it is mostly during the later stages (III/IV), when the ovarian cancer already have started to spread that the diagnostics is being made.[65,66,67,68,69] Once ovarian cancer cells are invasive, they can spread through both lymphatics, or via blood vessels to form metastasis in the parenchyma of the liver or lung. In addition ovarian cancer cells are also shed of from the ovary to form implants on the peritoneal surface, and this is made possible based on the anatomical structure, containing no barriers to prevent metastasis, in the peritoneal cavity.[70,71,72,73]

1.4 Epithelial-Mesenchymal transition (EMT)

Largely our bodies consist of epithelial and mesenchymal cells that have defined differences coupled to their physiological functions in the context of where they grow. Epithelial cells cover all outer and the most inner surfaces of tubular and glandular structures in the human body. Their functions include building a protective barrier against the outside environment, facilitate transport of substances across the epithelium, and mediate secretion in the glandular structures.[30,74,75,76] Mesenchymal cells, including fibroblasts and smooth muscle cells, are on the other hand located in stromal compartments of the body, and are separated from epithelial cells by the basement membrane. These type of cells need to be motile during tissue repair and remodeling, have supportive and contractile functions.[77,78,79]

Yet, during development epithelial cells trans-differentiate into mesenchymal-like cells through Epithelial-Mesenchymal transition (EMT). Cycles of EMT and the reverse mesenchymal-epithelial transition (MET) is needed for proper formation of the embryo.[5,80,81] However, EMT can also be reactivated in pathologies including cancer and fibrosis.[4,82] During EMT, epithelial cells lose apical-basal polarity and their cell-cell adhesion to neighboring epithelial cells, and convert to mesenchymal-like cells that are characterized by front-back polarity, which allows cells to migrate in a directional manner through extracellular matrix.[76,83] In cell culture, EMT is a visible process under magnification, and from growing in a cobblestone-like monolayer, epithelial cells undergoing EMT adopt a spindle-shaped mesenchymal morphology. At a molecular level, EMT induces reorganization of cytoskeletal proteins, loss of junction proteins and polarity complexes, and increased expression of MMPs with the capacity to modulate the extracellular matrix.[4,84,85]

Although EMT has been observed in many *in vitro* models of cancer cells, the significance of EMT during cancer progression and its relevance in human cancer has been debated, mainly because of lacking evidence of EMT in clinical cancer samples.[5,86] It has proved difficult to distingue cancer cells with mesenchymal properties from other mesenchymal cells in the tumor stroma. However, observations from the invasive front reveal

cancer cells, expressing both epithelial and mesenchymal markers, which are spreading from the tumor mass into the adjacent stroma. Besides from breast cancer this has been observed in different types of cancers including colon, cervical, thyroid and ovarian cancer.[87,88,89,90]

In addition, markers of EMT such as snail and slug have been shown to clinically correlate with disease relapse of both breast and ovarian carcinoma.[91,92] EMT has also been linked to basal-like, and metaplastic breast carcinoma and higher malignancies grades, again indicating that EMT worsen the clinical outcome.[93] Induction of EMT is induced by many different growth factors both during development and in cancer, and these includes include Notch, epidermal growth factor (EGF), hepatic growth factor (HGF), Wnt factors, and Transformer growth factor β (TGF-β). [94,95,96,97,98,99] Although several factors most likely cooperate to induce EMT in tumors, TGF-β stands out as a major EMT inducer in cancerous disease.[100]

1.5 Transformer growth factor beta (TGF-β)

There are more than 30 factors belonging to the TGF-β family and they can be divided into two branches, one including bone morphogenetic proteins (BMPs), anti-muellerian hormone (AMH), and growth and differentiation factors (GDFs), and the other consisting of nodal, lefty, activin, and TGF-β.[101,102,103] These factors are diversely expressed throughout development and in adulthood, to at a early stage e.g. promote differentiation of stem cells to regulate the body axis formation, and later maintain the homeostasis of the human body.[102,104]

Transforming growth factor beta ligands, TGF-β1, TGF-β2 and TGF-β3, are potent regulators of cell growth, differentiation and migration.[105] The different TGF-β isoforms signals through type I, II, III TGF-β receptors (TGFβRI, TGFβRII, and endoglin) to initiate downstream signaling pathways.[106,107] When TGF-β1 and TGF-β3 ligands are expressed and activated, through proteolytic cleavage or structural modifications in the extracellular matrix and at the cell membrane, they bind to TGFβRII. The activated TGF-β2 ligand is however dependent on the presence of the endoglin receptor to be able to bind TGFBRII with high affinity. When expressed, the TGF-β type 1 and type II receptors form homodimers in the endoplasmic reticulum (ER) and at the cell surface when ligands are not present. TGF-β ligands favor binding to TGFβRII homodimers forming a ligand-bound receptor complex that in turn has high affinity for binding to the TGFβRI receptors, resulting in an activated heteromeric signaling complex.[106,108,109] Activin receptor-like kinase 5 (ALK5) is the predominantly expressed TGFβRI in most cell types, and is activated by TGFβ binding to TGFβRII. Notably ALK5 signaling results in the activations of the transcriptional co-regulators SMAD2 and SMAD3, whereas ALK-1 or ALK-2 acitivate SMAD1, SMAD5 and SMAD8.[110,111,112]

The functional TβRII-TβRI heteromeric signaling complex is generally linked to human cancer, and it regulates the induction of downstream SMAD-dependent and SMAD-independent pathways.[113] Activation of this receptor complex, cause receptor-associated SMAD2 and SMAD3 to form homo and heterotrimeric complexes with the common mediator SMAD4. As these complexes forms they translocate to the nucleus where they regulate gene transcription and this is the canonical SMAD-dependent pathway of TGFβ signaling.[114,115] However, SMAD factors themself only bind DNA with low affinity, and therefore need to cooperate with other transcription factors in order to gain high binding affinity to specific target genes.[116] Various transcription factors, both activating and repressing gene expression are known to bind to the Smad complex, including forkhead, zinc-finger, bHLH, homeobox, and AP1 transciption factors.

In addition to the canonical SMAD signaling pathways, SMAD independent TGFβ signaling networks are also activated by the TGFβRI-TGFβRII heteromeric complex, including Ras homolog gene family, member A (RHOA), Rat Sarcoma protein (RAS), Rho family, small GTP binding protein (RAC1), phosphatidylinositol 3-kinase (PI3K), Mitogen-activated protein kinase, kinase, kinase 1 (MEKK1), and TGFβ activated kinase (TAK1) [117,118] Activation of all these kinases induce various signaling cascades with capacities to promote different cellular processes.[119,120]

1.5.1 Role of TGF-β in cancer

Under normal conditions, the basal release of TGF-β is sufficient to sustain tissue homeostasis.[109,121] Conversely, in damage tissue during injury, TGF-β is produced at high levels by cells in the stromal compartment, in order to control regenerative cell proliferation and inflammation.[122,123] It is important to know, that these conditions are activated in tumors as well, and that TGF-β is abundant in the tumor microenvironment. During early stages of tumor progression TGF-β act as a premalignant suppressor, while at later stages instead promote invasive and metastatic processes. In order to understand the dual nature of TGF-β signaling in cancer, one needs to consider the dynamic and contextual aspects of TGF-β induced pathways (Figure 2).[124,125,126,127]

Mutation in the genes encoding TGF-β receptors have been detected in cancer, but inactivation of TGFβRII in specific tissues rarely leads to spontaneous formation of tumors.[128] In the mammary epithelium, deletion of TGFBRII causes lobular-alveolar cell proliferation in mice [129], but no pathological changes in other types of epithelia.[130] Rather, it is during oncogenic stress or tissue injury that the role of TGF-β in restricting epithelial growth becomes evident, and depletion of TGFβRII or SMAD4 accelerates the malignant progression or neoplastic lesions with oncogenic stimuli.[131,132,133] This is observed in polyma virus middle-T (PyMT) oncogene driven mammary tumors, where deletion of TGFRII enhance carcinoma formation.[129] The cytostatic response to TGF-β is mediated through inhibition of the cell cycle in the GI phase, through mobilization of cyclin-dependent kinase inhibitors and c-Myc suppression.[134] In epithelial cells FoxO transcription factors targets and activate p15INK4b and p21CIP1 promoters, leading to inhibition of cyclinD-cdk4/6 complexes through p15Ink4b and cyclinE/A-cdk2 complexes by p21Cip1.[135,136]

As tumors progress the character of the TGF-β response changes, cytostatic effects are lost and instead TGF-β increase invasion and induce EMT.[137] As previously discussed, both intrinsic and extrinsic factors come into play when tumor cells turn invasive, and this is reflected in the TGF-β response. Many mediators of the non-canonical signaling cascade, downstream of the TGF-β receptor, are not exclusive to TGF-β signaling, but rather components utilized in different types of signaling systems. Thus, there are many signaling focal point that enable co-regulation of cellular responses between the TGF-β pathway and others induced by different growth factors in the tumor microenvironment. This is evident in the regulation of GSK-3β, known to inactivate EMT promoting transcription factors, and targeted for inactivation downstream of both TGF-β and Wnt signaling.[138] Enhanced induction, through combined stimulation of TGF-β and other growth factors such as EGF and HGF has been observed. EGF and HGF both induce RAS signaling that activate RAF/ERK/MAPKinase pathways, leading to loss of cytostatic response to TGF-β and activation of EMT promoting transcription factors such as snail and twist through induction

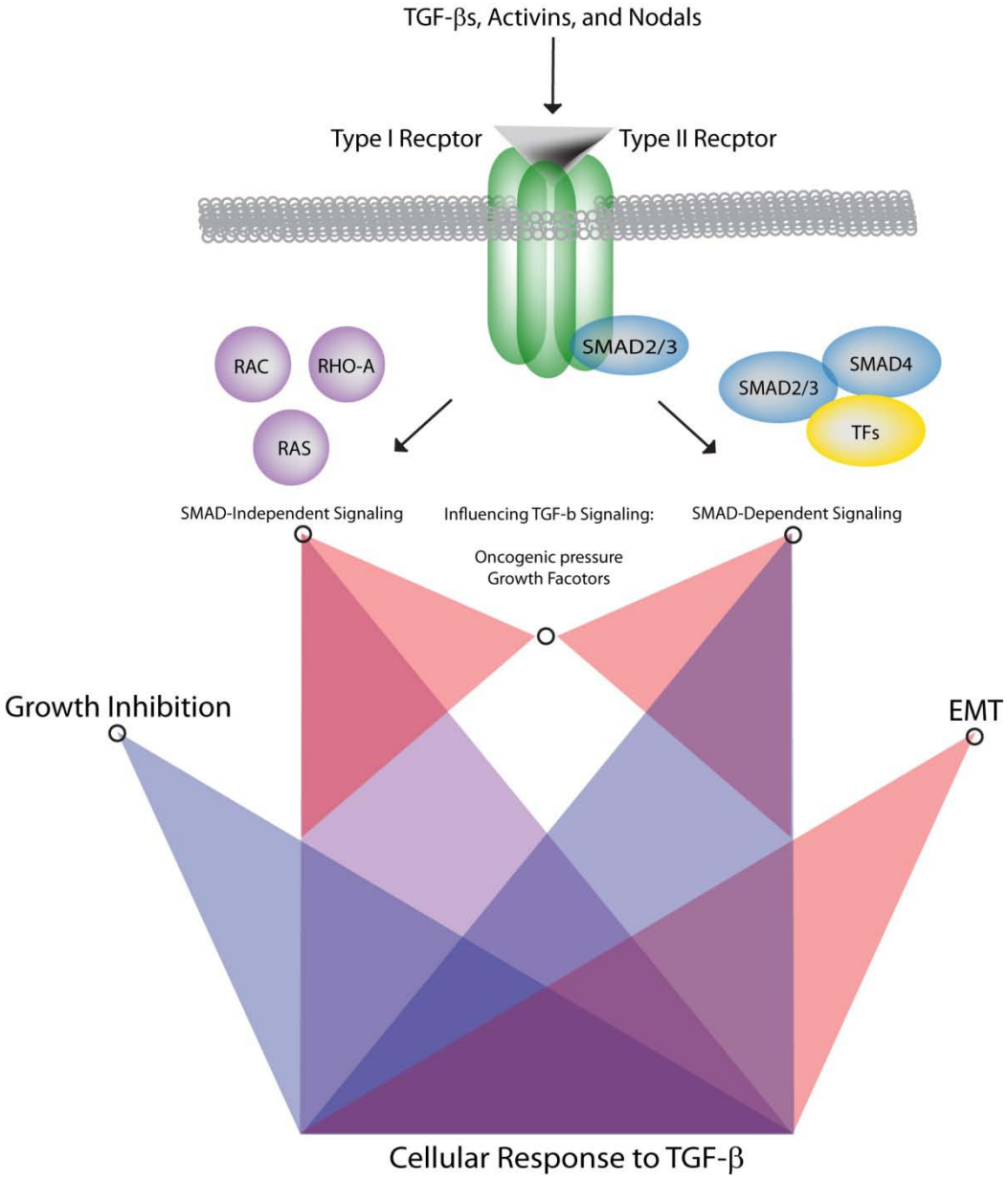

Figure 2. SMAD dependent and independent pathways of TGF-β signaling, subjective to intracellular influence in cancer cells, determine the cellular response to TGF-β. Activation of the heteromeric receptor complex of TGF-βRI and RII upon TGF-β ligand binding results in the induction of SMAD-dependent signaling, involving formation of the SMAD complexes with other transcription factors (TFs), and SMAD-independent signaling mediated upstream by e.g. RHO-A, RAC, and RAS. Both signaling cascades are under the influence of many intrinsic factors that are altered in tumor cells, and oncogenic pressure and other growth factors have the potential to alter the cellular response of TGF-β from growth inhibition to EMT.

of high-motility group A protein 2 (HMGA2).[139,140] Different members of the RAS protein family have been shown to induce EMT in various degrees.[139,141,142] In addition to other signaling pathways, non-canonical TGF-β pathway is also under influence of oncogenic activity, whereas abnormal activity of oncogenes such as, Pten, p53, and C-MYC are known to promote EMT.[143,144,145]

The dependence of SMAD complexes to interact with other transcription factors to bind and regulate target genes indicates that canonical TGF-β signaling is contextual.[113] Indeed, studies of TGF-β induced EMT has revealed several transcription factors interacting with the SMAD proteins to promote EMT, including both transcriptional repressors and activators. The outcome of Smad-dependent TGF-β signaling is therefore reliant on the transcription factors available in the target cell. In conclusion, EMT requires both that SMAD-independent signaling is altered due to oncogenic pressure or combined exposure from cytokines, and the right mix of SMAD interacting transcription factors in the nucleus of the target cell. [105,114,146]

1.6 Transcriptional regulation of EMT

Activation of TGFβ signaling in breast cancer cells during pro-metastatic conditions can, as discussed, induce EMT. In order to carry out the specific changes in the tumor cells when they turn mesenchymal, it is required that mechanisms involved in transcriptional regulation are altered – that genes previously turned off are turned on, and the reverse. In order to regulate gene expression different systems has been developed in the cell, involving regulation of the transcriptional complex by transcription factors, epigenetic modifications, and post-transcriptional regulation.[147] In transcriptional machineries, all of these factors work together to promote or maintain cellular functions and phenotypes. By now different transcription factors promoting EMT has been identified, including both transcriptional repressors and activators. Many of these factors have been shown to interact directly to co-SMADs and are thus activated during TGF-β induced EMT.[146,148]

During EMT transcriptional repressors will silence the expression of epithelial genes such as junction proteins. This is evident on the promoter of E-cadherin were Snail, Zeb and bHLH factors bind to repress the expression. [91,149,150,151] In the E-Cadherin promoter this is mediated through binding of these factors to E-box sequences with a 5´-CACCTG-3´core motif.[92,152,153] Binding of factors from the Snail family is generally thought to repress transcription through recruitment of chromatin remodeling complexes that silence the gene, although they can, in a context dependent manner, activate target genes as well.[154,155] Snail1 is a major promoter of EMT in development and cancer, and overexpression alone is enough to induce EMT in cell culture.[156,157] Snail overexpression has been shown to induce other repressors and is upregulated early during TGF-β induced EMT. In fact, a peak of Snail1 expression can be seen early after TGF-β stimulation in cultured epithelial cells.[158] Even if it is evident that factors of the Snail family regulate each other, these two aspects might suggest that Snail1 is essential to kick start EMT.

Transcriptional activators promoting EMT, on the other hand, will bind and increase the expression of mesenchymal genes and includes factors like B-catenin, AP-1, SP-1 and NFkB transcription factors. B-catenin, activated by Wnt signaling, interacts with TCF4/LEF transcription factors to induce genes involved in EMT, such as Vimentin and Snail2.[159,160] AP-1 promotes the expression of MMPs and is formed as heterodimers of jun-fos/fra proteins or as jun-jun homodimers and is known to promote invasiveness and metastatic capacity of cancer cells.[161] As an activator of Snail1 and Twist expression NFKβ overexpression is sufficient to induce EMT in mammary epithelial cells.[162]

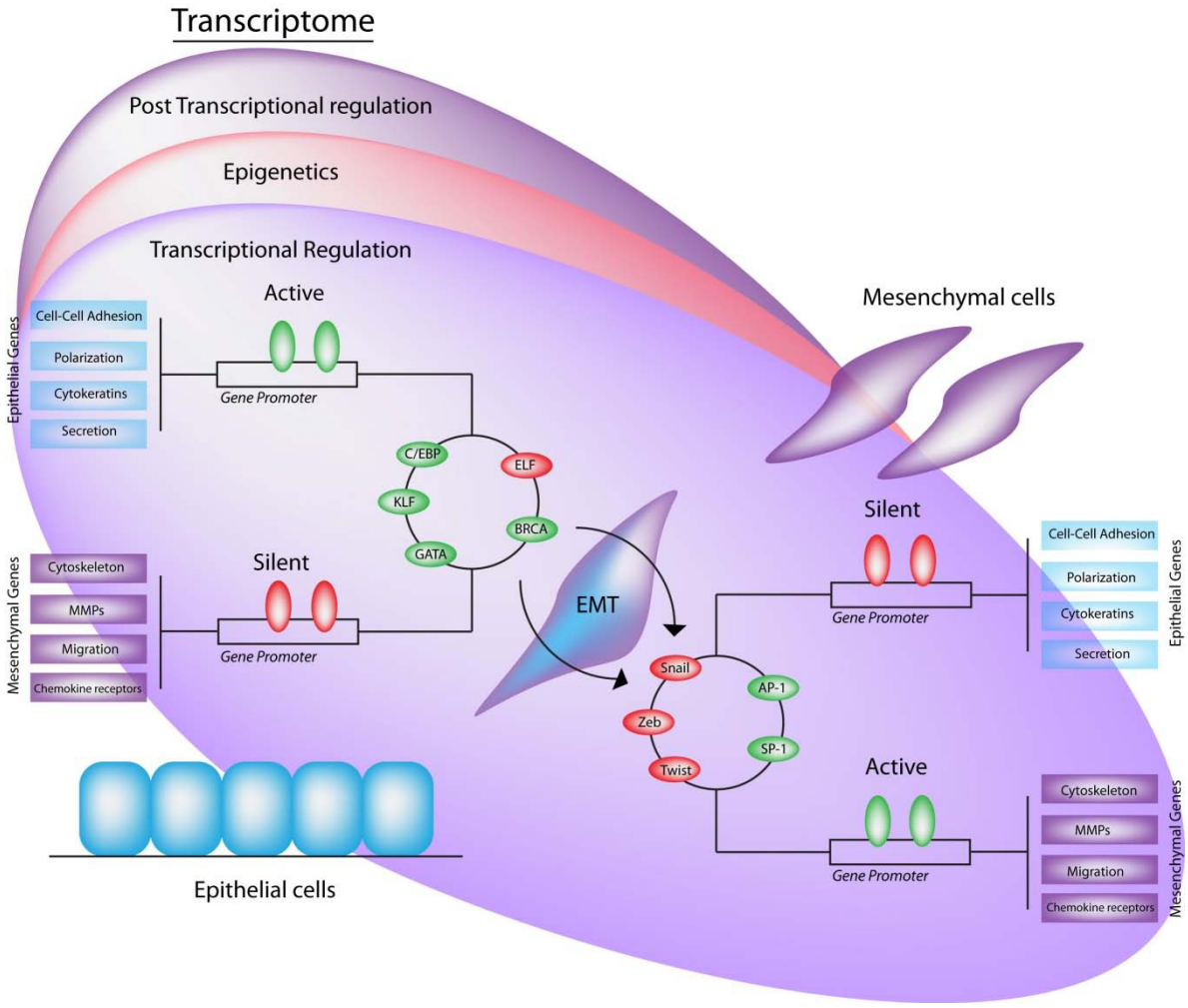

Figure 3. A transcriptional switch during EMT involves both epithelial and mesenchymal transcriptional activators and repressors. One central part of the transcriptome is transcriptional regulation. To maintain the epithelial phenotype a set of different genes needs to be kept active in the cell involving polarization, cell-cell adhesion, and in ducal structures secretion. As epithelial cells form transcriptional repressors also needs to inactivate expression of genes promoting a non-epithelial cell fate, including mesenchymal genes. Reversed circumstances are found in mesenchymal cells where genes involved in the mesenchymal cell functions are activated and other genes repressed. During EMT, factors such as TGF-β switch the transcriptional machine of the cell, turning off epithelial transcription factors and turning on mesenchymal. This leads to phonotypical changes promoting a migratory behavior of otherwise stationary epithelial cells.

Although the transcriptional inducers of EMT have been well characterized and studied there is less known about transcription factors able to prevent EMT. Even so, it is logical to assume that transcriptional programs activated to promote and maintain the epithelial phenotype needs to be inactivated during EMT. In line with this, different transcription factors known to promote epithelial differentiation during development have been shown to prevent invasive behavior of tumor cells and also EMT. Due to the initial epithelial programs in the tumor cells e.g. KLF, BRCA, ELF family members have been known to inhibit EMT through activation or repression of target genes. Still, further evaluation is needed to determine the capacity of epithelial differentiation factors to prevent and even revert EMT.[163,164,165,166,167,168]

1.6.1 CCAAT-enhancer binding protein beta

The CCAAT-enhancer binding protein (C/EBP) family transcription factors, consisting of 6 members (α-γ), are known to play crucial role in different cellular events including cellular proliferation, differentiation, metabolism and inflammation.[169] All factors within the C/EBP family contain the same leucine zipper domain at the C-terminus and they all share the capacity to bind to the CCAAT sequence of DNA. Although they all bind to the same sequence, the N-terminal part, which varies in size, of the C/EBP proteins enables different type of interactions and actions once bound to DNA.[170]

The expression of C/EBP proteins varies throughout different tissues of the human body, and in mammary epithelial cells C/EBPβ has been identified as differentiation factor towards luminal cell fate in the mammary epithelium and as regulator of branching morphogenesis.[170,171,172] Through alternative splicing C/EBPβ is produced in three isoforms, liver activation protein 1 and 2 (LAP1 and LAP2) both two transcriptional activators containing transactivation domains, and the shorter isoform liver inactivation protein (LIP). LIP is generally considered to act as a repressor of C/EBPβ activity since it lacks the transactivation domain.[173]

In breast cancer C/EBPβ has been described as deregulated, and in some cases inducing proliferation of tumor cells[174,175], but the role of C/EBPβ in cancer is not fully known. Different isoforms of C/EBPβ, and the ratio of LIP and LAP has shown to be of importance.[176,177] In line with this, inactivation of C/EBPβ activity due to high LIP/LAP ratio is a mechanism of evasion from the cytostatic TGF-β resonse in breast cancer. In this study C/EBPβ was shown to interact with Smad complexes to activate the expression of INK4b, encoding p15INK4b, a mechanism that was lost in metastatic breast cancer cells from pleural effusions.[178]

1.6.2 Forkhead box P4

Forkhead box (Fox) proteins, a family that all contain a common forkhead box DNA binding domain are evolutionally conserved transcription factors. In total there are 19 subfamilies with around 50 genes, and they play various roles in different biological processes during development including differentiation, proliferation and apoptosis. Certain members of the fox family of proteins have been linked to epithelial plasticity of cancer cells and deregulated expression of these proteins has been related to increased malignancy and metastatic capacity.[179,180]

Forkhead box P4 (FoxP4) found to be expressed in epithelial cells in different tissues, and have been described to play important roles in mediating differentiation.[181,182,183 184] Foxp4 is expressed in the pulmonary epithelium were it promotes differentiation of secretory epithelial cells through inhibition of goblet cell differentiation.[182] In neuroepithelial cells Foxp4 is known to transcriptionally repress N-cadherin in order to promote epithelial differentiation.[185] In mammary epithelial cells Foxp4 has been identified as a core transcription factor based on its enriched expression.[186] Although no studies have been performed specifically on the role of foxp4 in women´s cancer it has been shown to be a target for oncogenic translocation in breast cancer.[187] In addition, it has been identified as a susceptibility locus for prostate cancer, and decreased levels of Foxp4 has been reported in kidney cancer.[184,188]

1.7 Tight junctions

Epithelial cell layers form barriers that protect multicellular organisms from the external environment. Epithelial integrity is maintained by tight junctions, adherens junctions and desmosomes. Adherens junctions are formed by E-cadherin,[189] while transmembrane components of tight junctions include claudins, occluding,[190] and Ig-like proteins like the coxsackie- and adenovirus receptor (CAR), the junction adhesion molecules (JAMs) and CAR-like mambrane protein (CLMP).[190,191,192] Tight junction proteins such as occludins, claudins and the recently identified members of the large Ig superfamily, e.g. CAR, are all mediators of cell-cell adhesion. Tight junctions affect many cellular functions, including cytoskeletal dynamics, polarized vesicle trafficking and proliferation.[193] TJs control physiological flux of ions and solutes across the barrier and are regulated by different growth factors, cytokines and hormones.[194]

Transmembrane components of TJs are located to the apical side of the cells facing the lumen, and binds to scaffolding proteins that have an intracellular location close to the membrane. In turn scaffolding proteins are bound to the actin filament, and together this forms a netlike structure that spans across the epithelium constituting the TJ barrier.[195,196,197,198] Interestingly, many kinases, phosphatases and transcription factors have been described to localize at the TJs, indicating that not only do TJs regulate extracellular conditions and events, but also serve as a hub for intracellular signaling.[199,200] Certain proteins, such as RAC, and protein kinase C (PKC) are recruited to the TJs to perform their functions, [201] others like transcription factors c-jun and c-fos are inactively stored at the TJs. [202] Upon deregulation or disruption of the TJ barrier factors belonging to the TJ hub are both activated and inactivate, possibly as a mechanism for the cell to quickly response to changed conditions.[201,203]

1.5.1 Coxsackie- and adenovirus receptor (CAR)

The coxsackie- and adenovirus receptor (CAR) is a member of the large immunoglobulin-like receptor family and a component of epithelial tight junctions and was first identified as a receptor for coxsackie B and type C adenoviruses. [204,205] Consequently, along with some other TJs, CAR serve as an entry point for viruses.[206] TJs all share the ability to promote cell-cell adhesion, and many of the TJ components have been proven to be dispensable. CAR on the other hand is, together with a few other TJs, vital both during development and in adult mice. Depletion of CAR in development leads to malfunction of the heart, as CAR mediates contact between the discs of cardiomyocytes.[191,207] Knock down of CAR in adult mice leads to dilation of the instestinal tract, acinar-to-ductal metaplasia of the exocrine pancreas, and cause lethality.[208] CAR is downregulated early during TGF-β induced EMT, in a similar

fashion to E-cadherin by master EMT regulators, but the role of CAR in the EMT response is not clear.[209]

1.8 Treatment of women´s cancer

One of the major hallmarks of cancer is the evasive behavior of tumor cells towards growth inhibitory and an apoptotic stimulus compared to normal cells, and was recognize early on in the field of oncology.[3] In the mid 20th century it was discovered that alkylating agents originally derived from mustard gas, had the capacity to target fast dividing tumor cells, and once injected into patients with leukemia had a beneficial outcome increasing survival.[210] This kind of treatment, now including several agents essentially all targeting fast dividing cells, was termed chemotherapy, and is commonly used to treat patients with different types of cancerous diseases worldwide.[211]

In addition to chemotherapy, both radiation therapy, and removal of the tumors through surgery is the standard line of treatment for cancer.[212,213] In ovarian cancer the bulk tumor is removed through cytoreductive surgery, which means that the entire visible tumor is removed and followed by adding heated chemotherapy drugs to the operated area. Breast tumors are removed surgically and followed up with chemotherapy afterwards. Based on the clinical features of tumors, a scheme and agents for chemotherapy is adopted individually for patients of breast and ovarian cancer.[51,214] The commonly used drugs for breast cancer treatment include the anthracyclines doxorubicin and epirubicin, and the taxanes paclitaxel and docetaxel. In some cases these are used in combination with other drugs, like fluorouracil (5-FU), cyclophosphamide, and carboplatin. For ovarian cancer the standard approach is to combind plantinum compounds such as cisplatin or carboplatin with taxane drugs like paclitaxel.[215,216]

Cisplatin, the drug studied in this thesis, cause antitumoral effects through its ability to for DNA breaks and pronounced DNA damage, mainly in proliferative cells. Compared to carboplatin, there are more side effects in non proliferative tissues associated with cisplatin, including oto-, nephro and neurotoxicity.[217,218] Besides from DNA damage, cisplatin induces apoptosis through non-nuclear targets, with mitochondria as a major one. This is due to the acute effects of cisplatin inducing reactive oxygen species (ROS) and binding to mitochondrial DNA (mtDNA).[219]

In ovarian cancer, 70% of the patients will respond to platinum- and taxane-based chemotherapy after surgery, and in 50% of these patients no cancer can be detected after 5 month of treatment. In breast cancer approximately 20% of treated patients have a relapse over a 10 year period. Although the reduction of visible tumor is present in EOC, 75% of all patients will relapse within 3 years. For triple negative breast cancer 20% of patients will relapse with signs of distant metastasis.[6,220,221] In EOC and breast cancer recurrent cancer cells are difficult to treat since they have developed multiresistency towards chemotherapy.[82,222] These facts indicate that further alternative approaches for cancer treatments are necessary in order to cure patients from the disease. As the field of cancer research is progressing, and other hallmarks of cancer besides cell proliferation and growth are being explored, many new therapies are under development e.g. immunotherapies and anti-angiogenic treatments.[223,224,225]

2 AIMS OF THE STUDIES

- To charachterize ovarian cancer cells resistant to cisplatin treatments in terms of EMT and cancer stem cell properties

- To Study the role of C/EBPβ, as a transcriptional activator of junction proteins, in TGF-β induced EMT in breast cancer

- To investigate role of Foxp4, as a transcriptional repressor of mesenchymal genes, in TGF-β induced EMT in breast cancer

- To study the role of CAR in regulating the EMT response to TGF-β in breast cancer cells

3 RESULT AND DISCUSSION

3.1 Paper I

Repeated cisplatin treatment can lead to a multiresistant tumor cell population with stem cell features and sensitivity to 3-bromopyruvate

Chemotherapies have mostly been developed to target the cell proliferative behavior of tumor cells. However, EOC cells frequently get resistant to this kind of therapy and it is still not clear to which features of the EOC cells that resistance is coupled to, but data indicate that cisplatin resistant cells have more aggressive properties. The aims of this study was therefore to (i) investigate whether EOC cells induced to become resistant to cisplatin treatment developt EMT and cancer stem cell characteristics, and (ii) study alteration in mitochondrial status of these cells, since cisplatin has non-nuclear target effects.

First, human ovarian carcinoma, SKOV-3, cells were repeatedly treated with cisplatin for long term, to generate resistant (R) cells, or treated with EtBr to make cells deficient in mitochondrial DNA (ρ0). To verify chemoresistanant features, SKOV-3-R cells were treated with other platinum drugs including carboplatin, paclitaxel and 5-FU. Based on IC-50 values after 72 h of treatment, SKOV-3-R displayed many fold increased resistance to these drugs compared to parental SKOV-3 cells. These data confirm that the repeated cisplatin treatment generated multi resistance cells. In contrast SKOV-3-ρ0 cells did not display increased resistance. Known genes for drug resistance, Bcl-2, and the efflux protein ABCG2, was not upregulated in SKOV-3-R cells.

Leading up to this collaborative effort, was the finding that SKOV-3-R cells displayed a more mesenchymal morphology, and my part was primarily to assist further investigation of this finding. Western Blot analysis showed decreased levels of E-cadherin and increased levels of Vimentin and Twist in SKOV-3-R cells compared to parental and ρ0 cells. Immunoflourescent staining confirmed increased nuclear staining of Twist and Snail in SKOV-3-R cells, suggesting that these factors might contribute to induction of the EMT phenotype in resistant cells. Using an automated xCelligence® device, motility of SKOV-3-R cells was evaluated, and in comparison to parental cells, displayed greater motility.

Since a link between EMT and cancer stem cells (CSC) has previously been described,[226] we were also interested to study expression of CSC markers and the capacity to form mammospheres in SKOV-3-R cells. In line with this, western blot analysis showed upregulation of known CSC markers CD44, CD117 and ALDH1 in SKOV-3-R cells compared to parental and ρ0 cells, but not the stem cell markers Oct-4 and Nanog. Functional sphere forming assays, on low binding cell culture plastics, showed that SKOV-3-R had higher self-renewal capacity and compared to parental and ρ0 cells did not lose viability over time. These data suggest that multi-resistant ovarian cancer cells after cisplatin treatment show CSC and EMT characteristics.

Based these findings, we were interested in investigated ways to target the stem cell and EMT features of the SKOV-3 cells in order to decrease cellular viability to drug treatment. Since tyrosin kinase receptor CD117 was one of the more upregulated markers in SKOV-3-R cells this was our first target. CD117 is a blocked by imatinib mesylate (gleevec), thus SKOV-3-R cells were treated for 48 h with the gleevec singly and in combinations with cisplatin, whereafter they were allowed to recover in drug-free medium for another 72 h. In

these experiments imatinib mesylate, alone or in combination, had no effect on growth at any time point in SKOV-3-R. Similar negative results were obtained with ISCK03 (5 μM), another inhibitor of CD117 that did not lower the IC_{50} value for cisplatin in SKOV-3-R cells..

Taken together these results indicate that targeting CD117 did not restore toxicity to cisplatin in SKOV-3-R cells. Another feature of the SKOV-3-R cells discovered throughout the project was that the mitochondrial status was changed, and SKOV-3-R cells had increased mitochondrial mass compared to parental cells detected with potential-independent MitoTrackerGreen dye. This was supported by increased levels of the nucleus-encoded mitochondrial proteins voltage-dependent anion channel (VDAC) and cytochrome c. Since mitochondrial metabolism and cell death is known to be regulated by VDAC, partially through binding of hexokinase-II (HK-II), we further investigate HK-II levels in the SKOV-3-R cells. Indeed we found upregulated levels of HK-11 in SKOV-3-R cells, and to a larger extent in SKOV-3-ρ_0 cells, which makes sense since they are devoid of ETC and respiration. 3-bromopyruvate (3-BP) is small molecule that a have been shown to have antitumoral effects by targeting and inducing dissociation of HK-II, and we were curious to evaluate if SKOV-3-R was sensitive to this compound. Treatment with 3-BP in one dose for 48 h was enough to reduce proliferation in both partental and SKOV-3-R cells, however while parental cells could resume to normal growth under drug-free conditions during the next 72 h, SKOV-3-R cells was unable to. In addition, we found that 3-BP enhanced the antiproliferatvie effects of cisplatin.

Combination of drugs targeting different features of tumor cells and their environment, have shown promise to treat cancer, and this study belongs to this category of studies. In the described case, abnormal metabolic activity of HK-II in SKOV-3 cells, made them vulnerable to its inhibitor 3-BP. However in our attempts to target EMT and CSC features of the SKOV-3 cells, through CD117 inhibition, we had no effect. Further exploration and understanding of these processes in tumor cells might therefore be needed to be successful in such an endeavor.

3.2 Paper II

MiR-155-mediated loss of C/EBPβ shifts the TGF-β response from growth inhibition to epithelial-mesenchymal transition, invasion and metastasis in breast cancer.

As previously discussed, multiple factors are involved in tumor progression and the induction of EMT, and needs to be taken into consideration in studies conducted on EMT. Although this is rational, TGF-β stands out as a major inducer of EMT and is frequently associated with EMT both *in vitro* and *in vivo* in cancer and development. Interestingly, TGF-β signaling has opposite roles in cancer, acting as a tumor suppressor at early a stage, and as a promoter of malignancy and EMT at a later stage. Data also suggest that the switch in TGF-β response actually revolves around factors involved in EMT, and the TGF-β switch might be a premise for EMT in cancer. Following this line of though, and based on its previously described role in the cytostatic effects of TGF-β and being a differentiation factor in mammary epithelial cells, C/EBPβ emerged as interesting transcription factor to study in EMT, and in paper II we aimed to test the hypothesis that loss of C/EBPβ has a preventative role against TGF-β-induced EMT in breast cancer.

In order to study C/EBPβ expression in breast cancer cells with a less differentiated phenotype potentially undergoing EMT, we preformed immunoflourescent staining on human breast carcinoma samples and did subsequent confocal microscopy analysis. E-Cadherin, known to be lost in invasive cancer and common marker for EMT, was stained in combination with C/EBPβ. In breast cancer samples of well-differentiated

ductal carcinoma *in situ* (DCIS), strong staining of E-cadherin was detected at cell–cell junctions, and pronounced nuclear C/EBPβ staining was detected in the breast cancer cells. In a series of eight invasive ductal breast carcinomas, which had been classified according to their status of estrogen receptor, progesterone receptor and HER2, we next analyzed the expression of E-cadherin and C/EBPβ. We found decreased expression of E-cadherin in areas of triple-negative tumors, and, in total, lower levels of E-cadherin in triple-negative tumors compared to tumors positive for estrogen receptor, progesterone receptor and HER2, or only HER2. Nuclear staining for C/EBPβ was reduced in triple-negative tumors and linear regression analysis revealed a significant correlation (P=0.0011) between the expression of E-cadherin and C/EBPβ in all tumors analyzed. Further analysis demonstrated significantly decreased expression of C/EBPβ in E-cadherin-negative areas compared with E-cadherin-positive areas of the triple-negative breast tumors. In support of these finding we could in a mouse model of mammary cancer progression, with mice overexpressing the polyoma virus middle T antigen under the mouse mammary tumor virus promoter (MMTV-PyMT mice), find that C/EBPβ expression was lost at an advanced stage of cancer at the age of 10 to 14 weeks. Co-staining for E-cadherin and C/EBPβ in tumor sections from 10- and 14-week-old MMTV-PyMT mice showed loss of C/EBPβ expression in E-cadherin-negative areas compared with E-cadherin-positive.

To explore the mechanisms of C/EBPβ loss in breast cancer, we used an established model of TGF-β1-induced EMT in namru mouse mammary gland (NMuMG) epithelial cells. Treatment with TGF-β1, induce visible signs of EMT after 24 h in NMuMG cells, and at 48 h detectble changes in protein level of epithelial markers such as E-cadherin and gain mesenchymal markers e.g. Vimentin are present. Immunoflourecent analysis of untreated NMuMG cells showed typical nuclear staining of C/EBPβ, which was lost in cells treated with TGF-β1 to undergo EMT. Further western blot analysis of C/EBPβ showed that all three C/EBPβ isoforms (LAP1, LAP2 and LIP) were decreased in TGF-β1-treated NMuMG cells, and no obvious change in the ratio between LIP and LAP was detected. By comparison, TGF-β1 treated mouse mammary EpH4 cells, lacking the capacity to undergo EMT, did not induce loss of C/EBPβ, suggesting that loss of C/EBPβ is an EMT specific event.

Interestingly C/EBPβ was not downregulated at RNA level indicated by qPCR analysis, suggesting post transcriptional regulation of C/EBPβ during EMT. Based on other studies, showing that Cebpb mRNA is a target of miR-155 in B-cells and macrophages[227,228], and that miR-155 is a known oncomiR in cancer [229], we hypothesize that miR-155 might be a repressor of C/EBPβ during TGF-β1 induced EMT in breast cancer. To test this, NMuMG cells were transfected with a synthetic miR-155 inhibitor (50 nM), in combination with TGF-β1 treatment to study whether the presence of the miR-155 inhibitor would rescue C/EBPβ expression. We found that in the presence of the miR-155 inhibitor, the expression of C/EBPβ was increased at baseline, and less repressed during EMT. On the contrary, transfection of NMuMG cells, with a synthetic miR-155 mimic (50 nM), resulted in decreased levels of C/EBPβ at baseline, and further repression after TGF-β1 treatment. Further analysis by qPCR showed that the expression levels of miR-155 were approximately 4.5-fold higher in NMuMG cells compared with EpH4 cells at baseline, and increased in NMuMG cells subjected to TGF-β1. This experiment also shows that the expression level of C/EBPβ (all isoforms) was considerably higher in EpH4 cells compared with NMuMG cells.

To study whether miR-155 mediated loss of C/EBPβ could sensitize breast cancer cells to TGF-β1 induced EMT, we set up a series of gain and loss of function experiments. Lentivirus mediated knock down of C/EBPβ through overexpression of small hairpin RNA (shRNA), lead to reduced baseline expression of E-cadherin and CAR, and potentiated TGF-β1 induced EMT, indicated by increased levels of vimentin and further suppression of E-cadherin and CAR expression. Conversely, transient overexpression of LAP2 in

NMuMG cells resulted in increased mRNA levels of *Cdh1* and *Cxadr* at baseline, and less repression of both genes in response to TGF-β1 (2 ng/ml, 24 h).

Based on these data we wanted to examine the potential of C/EBPβ to revert cells maintaining an EMT program back to the epithelial phenotype. In order to do so we treated NMuMG cells with TGF-β1 for more than 14 days (long-term), which had been reported to induce robust EMT and evasion from the cytostatic effects of TGF-β. Next we transiently overexpressed LAP2 in these cells, which lead to increased protein levels of epithelial markers E-Cadherin and CAR. On the contrary, overexpression of LAP2 did not influence the expression of mesenchymal EMT markers, including master regulators such as Zeb1, Slug, Snail and Twist. Together, these data indicate that reactivation of C/EBPβ only partially revert EMT cells back to their epithelial origin.

Next we wanted to study if C/EBPβ levels in breast cancer cells would also affect the invasive behavior *in vitro* and the metastatic process *in vivo*. To do so, we used the 4T1 mammary tumor model, a TGF-β driven model of metastasis, and preformed loss of function experiments. Lentivirus mediated knock down of C/EBPβ resulted in less expression of E-cadherin and increased expression of vimentin, and shCebpb expressing cells were more invasive in boyden chamber assays towards a TGF-β1 gradient. We went on injecting 4T1 cells expressing shControl or shCebpb subcutaneously into the flank of syngeneic BALB/c mice, tumors were formed and surgically removed after 2 weeks. Measurements of tumor growth indicated that tumors with repressed cebpb expression grew slower compared to control, but two month after tumors were surgically removed had formed more metastasis in the lungs.

Our results indicated that C/EBPβ levels could alter the outcome of TGF-β signaling promoting EMT, invasion and metastasis in breast cancer, and we were curious to study the underlying mechanisms further. Our results had shown that knock down C/EBPβ was enough to reduce expression of E-cadherin and CAR, which lead us to hypothesize that C/EBPβ can modulate the EMT response of TGF-β by acting as a transcriptional activator of junction proteins.

In order to address this hypothesis, computer-based software was used to scan for putative binding sites for C/EBP transcription factors in the genomic DNA promoter sequences of several genes encoding junction proteins, and sites were found in the promoters of following genes: *Cdh1*, *Cxadr*, *Cldn3*, and *Ocln*. Further, chromatin immunoprecipitation (ChIP) assays showed specific C/EBPβ binding to the regions of putative binding sites in the promoters of the *Cdh1* and *Cxadr* genes in NMuMG cells. Since C/EBPβ had the capacity to bind to these two promoters we performed promoter reporter assays to determine if C/EBPβ also could activate them. These experiments showed that overexpression of C/EBPβ activated the *Cdh1* promoter by twofold and the Cxadr promoter by 2.5-fold and that overexpression of LAP2 by itself was more potent than C/EBPβ to activate the promoters. Knock down of C/EBPβ resulted in less mRNA expression of cdh1 and cxadr, while overexpression induced the expression. Treatment of TGF-β1 to induce EMT, also caused C/EBPβ to dissociate from the *cdh1* and *cxadr* promters.

This study, in combination with the previous study on cytostatic response, suggests that C/EBPβ, a transcription factor promoting epithelial cell differentiation, have the capacity to inhibit EMT and restore the TGF-β response towards growth inhibition. Even so, C/EBPβ lacked the capacity to fully revert EMT, which might indicate that reversion of EMT, based on transcriptional regulation, demands altered action of other transcription factors as well.

3.3 Paper III

Foxp4 controls EMT in breast cancer cells by acting as a transcriptional repressor of Snail1

During the course of our studies we identified Foxp4, a novel member of the family of Forkhead box (Fox) P transcription factors, as a C/EBPβ target gene in mammary epithelial cells (data not shown). Based on this, and recent published findings showing that Foxp4 is a repressor of N-cadherin in neuroepithelial cells, we initiated studies to elucidate whether Foxp4 could play a role in regulating EMT in breast cancer.

First we took a bioinformatical approach and performed meta-analysis of publically available gene data sets on breast cancer and normal mammary gland material, and we followed up with immunohistochemistry analysis on invasive breast cancer patient samples. The meta-analysis revealed that Foxp4 expression was decreased in invasive breast carcinoma compared to normal mammary gland, and a weak positive correlation between E-Cadherin expression and Foxp4 in invasive breast carcinoma samples. Immunohistochemical staining also supported these results showing that nuclear Foxp4 expression was decreased in invasive breast cancer and that cells expressing lower levels of E-Cadherin had significantly decreased levels of Foxp4.

These data suggested that foxp4 expression is lost in invasive cancer compared to normal mammary epithelium, an indication that maybe oncogenic conversion of epithelial cells is a causative effect of foxp4 loss. In particular, oncogenic variants of RAS have been linked to malignant progression and EMT of breast cancer cells. Thus, in order to study foxp4 expression during tumor progression driven by oncogenic HaRAS, we used a transgenic mouse model of inducible MMTV-HaRAS expression in Balb/c mice. Mice of this model develop mammary tumors that progress into invasive tumors with signs of EMT properties, and metastasize to the lungs. As tumors formed after 1-3 weeks of HaRAS activation, we observed downregulation of Foxp4 expression in the nuclei of cancer cells, and after 4 weeks of tumor progression we had close to non-detecteble levels of Foxp4 in the tumor cells. To study whether HaRAS activation directly affects FoxP4 expression, we used adenovirus vectors to transiently overexpress HaRAS and KRAS in mouse mammary epithelial, EpH4, cells. Overexpression of either HaRAS or KRAS for 48 h resulted in decreased expression of FoxP4, both at mRNA and protein levels.

In line with the studies performed on C/EBPβ, we wanted evaluate if foxp4 could impact TGF-β induced EMT, something that was also supported by our findings that foxp4 expression was decreased in invasive human breast cancer cells with signs of EMT. Again we used NMuMG cells, and induced expression of Foxp4 shRNA through lentivirus transduction. Knockdown of Foxp4 by itself was enough to induce an E-to-N-cadherin switch in NMuMG cells, and cause increased levels of vimentin. Further, treatment with TGF-β1 for 48 h resulted in a more pronounced EMT phenotype in shFoxp4 compared to shControl expressing NMuMG cells. Knock down of Foxp4 also lead to increased invasive behavior of NMuMG cells in the boyden chamber assay, were cells migrated in matrigel towards a TGF-β1 gradient.

As an additional experiment to further study the implications of reduced Foxp4 expression during EMT, we did knocked down of Foxp4 in mouse mammary EpRas tumor cells. Once injected into syngeneic BALB/c mice, these cells have the capacity to undergo EMT. Knockdown of Foxp4 in EpRas cells resulted in increased expression of N-cadherin and vimentin, reduced expression of E-cadherin and CAR, and increased invasive capacity *in*

vitro. Although knock down of Foxp4 did not affect tumor growth, immunofluorescence staining indicated that cells in tumors formed by shFoxp4 expressing cells expressed lower levels of E-cadherin and higher levels of N-cadherin compared to shControl tumors. Together, these results support the hypothesis that that loss of Foxp4 promotes EMT in both normal and transformed mammary epithelial cells.

Since Foxp4 is a known transcriptional repressor, but reverted both mesenchymal and epithelial genes during EMT, we hypothesized that Foxp4 transcriptionally repress one or several master regulators of EMT. To test this, we analyzed the expression of a set of master regulators including Snail1, Snail2, and Zeb1 in NMuMG cells after Foxp4 knock down. In NMuMG cells expressing shFoxp4, mRNA levels of Snail1, Snail2, and Zeb1 were increased compared to control shRNA. To evaluate if all these genes were directly repressed by Foxp4, or if the reduction was due to secondary effects, we instead transiently overexpressed Foxp4 and analyzed the mRNA expression at earlier time points. At a 24 h time point of Foxp4 overexpression no significant changes could be detected in mRNA levels for any of the genes, however at 48 h both *cdh2* and *snai1* were significantly repressed but not *snai2* and zeb1.

As these results suggested that Snail1 might be a direct target of Foxp4, we did sequence analysis on the 1000 base pair promoter sequence located upstream of the first ATG of the mouse Snai1 gene, revealing three putative FoxP binding sites. Transfecting cells with luciferase reporter constructs containing full length or truncated versions (575, 300, 100 bp) of the *snai1* promoter in combination with Foxp4 overexpression vector resulted in repression of the full length *snai1* promoter. The repression by Foxp4 was only rescued when all three putative binding sites were truncated. In addition, ChIP assays verified binding of Foxp4 to the regions of these 3 putative binding sites in the *snai1* promoter in NMuMG cells.

Knock down of Foxp4 in NMuMG cells resulted in increased mRNA levels of Snail1 at baseline compared to control cells and based on these results we next asked whether Snail1 also would be differently expressed during EMT in Foxp4 depleted cells. Following treatment with TGF-β1 for 24h the induction of Snail in cells with impaired Foxp4 expression was robustly increased compared to control cells. At protein level the difference could be observed between control and Foxp4 knock down cells after 48 h TGF-β1 treatment, however no detectable levels of Snail1 was found at baseline in both control and knock down. These data may indicate that increased RNA levels of Snail1 alone is not enough to increase protein levels of Snail1, and suggest that post transcriptional stabilization of Snail1 also needs to occur, something that is known to happen during TGF-β1 induced EMT. Finally, immunohistochemical staining of human breast cancer samples showed increased staining of Snail1 in Foxp4 negative areas of tumors, perhaps implementing a clinical relevance of this mechanism since Snail1 is linked to a worse clinical outcome.

As a next step we were interested in investigating if reactivation of Foxp4 in breast cancer cells could revert cells back from EMT. Transient overexpression of Foxp4 in NMuMG cells resulted in slightly reduced levels of N-cadherin and vimentin, both at baseline and upon TGF-β1 treatment for 24 h. In addition, downregulation of E-cadherin and CAR was partially rescued after TGF-β treatment in Foxp4 overexpressing cells compared to control. These data suggest that Foxp4 can inhibit induction of EMT. Next, we wanted to determine whether overexpression of Foxp4 could revert EMT in human breast cancer cells. For these studies, we used invasive human breast cancer MDA-MB-231 cells, which are known to display EMT properties. Stable, lentivirus-mediated overexpression of Foxp4 in MDA-MB-231 cells resulted in reduced expression of Snail1, N-cadherin and Vimentin, and increased expression of CAR. E-cadherin was not detected in these cells. These results indicated that overexpression of Foxp4 was sufficient to induce MET in human breast cancer

cells. In line with this, we found that Foxp4 overexpressing MDA-MB-231 cells were less invasive in the boyden chamber assay towards a TGF-β1 gradient.

Based on our results, it will be of further interest to evaluate if Foxp4 overexpression in MDA-MB-231 and other invasive breast cancer cells will influence the course of tumor progression and metastatic process *in vivo*. Such experiments would be necessary in order to draw further conclusions about the role and significance of Foxp4 loss in breast cancer.

3.4 Paper IV

The Coxsackie- and Adenovirus Receptor Controls Akt Signaling and EMT in Breast Cancer Cells by Regulating Pten Localization and Stability at Tight Junctions

Study II and III both indicate that loss of transcription factors that normally promote epithelial differentiation is important for the induction of EMT. In this study we wanted to determine whether changes in tight junctions, which are closely linked to and essential for epithelial differentiation, play a role in EMT. In particular we were interested in CAR, since it is an essential TJ in adult mice. We set out to test the hypothesis that CAR could be involved in regulating the TGF-β response.

To test this hypothesis, we used EpRas and EpXT cells to perform gain and loss of function experiments. Knockdown of CAR in EpRas cells using siRNA did not affect expression of E-cadherin, Occludin and Vimentin after transfection, however with TGF-β1 treatment further repression of both E-cadherin and occluding, and more induction of vimentin was induced in CAR knock down cells. In addition CAR knockdown lead to increased invasion towards a TGF-β1 gradient in the boyden chamber assay. Overexpression CAR in EpXT cells for 48h on the other hand, resulted in increased expression of both E-cadherin and occludin, and decreased expression of Vimentin. EpXT cells overexpressing CAR were also less invasive compared to control cells. These data confirmed the hypothesis that CAR could regulate the EMT response in breast cancer cells.

As previously discussed tight junction act as a hub for intracellular signals, and this lead us on to study if CAR knock down could change known signaling pathways downstream of the TGF-β receptor. Western blot analysis on CAR knockdown versus control cells indicated that phosphorylation of Smad3 (pSmad3), p38 (p-p38) and ERK1/2 (p-ERK1/2) did not change neither at baseline nor after TGF-β1 treatment for 1 h. On the other hand, phosphorylation of Akt (p-Akt) was more prominent in CAR knockdown cells than control cells, both at baseline and after TGF-β1 treatment. Further, phosphorylation of Akt downstream target GSK-3β was increased in CAR knockdown cells, and immunoflourescent staining of knock down cells indicated increased levels of nuclear Snail and Twist1. These results suggest that the branch of Akt signaling inducing EMT is activated in cells with impaired CAR expression.

Our next aim was to determine how CAR regulates Akt signaling in the cells. Interestingly, the phosphatase Pten, has been described to partially localize to tight junctions, and it is together with PI3K the major regulator of akt signaling. Pten localization to the TJs is mediated by interactions with the pdz-binding motif in the scaffolding proteins of MAGI protein family. One of the MAGI proteins, namely MAGI1, is also describe to interact with CAR, an interaction that is important for the recruitment and stabilization of MAGI1 to the TJs. [230,231] Based on these notions we aimed to investigate if CAR could stabilize Pten at TJs, something that would suppress Akt signaling. Immunoflourescence analysis on mammary EpH4 cells, with the capacity to polarize, revealed that both Pten and CAR co-locilezed at the TJs together with Magi1. Supporting physical interactions between these three proteins, co-immunoprecipitation (Co-IP) experiments, using a MAGI1 antibody, precipitated both CAR

and Pten in EpH4 cells. Since CAR is lost gradually at TJs during TGF-b1 induced EMT we were interested to study if a similar pattern would be seen with Pten and Magi1. Indeed when we induced EMT for 48h in EpRas cells we found that all three proteins were mainly lost at TJs and that only a dot-like staining remained. Neither could Pten be detected at the TJs in EpXT cells which are stably in EMT. Further siRNA mediated knock down of CAR resulted in increased phosphorylation of Pten, which is known to cause destabilization of the protein, and lower total protein levels of Pten. Conversely overexpression of CAR lead to less phosphorylation of Pten and increased total protein levels. These data indicate a role of CAR in stabilization of Pten at the TJs. Based on this, we finally treated CAR knock down cells with a PITenin, a PIP3 antagonist, to verify if Akt activation in CAR knock down cells is bound to the activity of PIP3 conversion by Pten. Inhibition of PIP3 in CAR knock down cells partially reversed the Akt activation in siCAR expressing EpRas cells.

This study indicates that cell differentiation goes beyond transcriptional regulation and that the other parts of the epithelial phenotype, in this case CAR expression, play a part in maintaining the epithelial phenotype during EMT. As previously discussed, there are hubs were the TGF-β pathway is connected with other signaling pathways, but the multi molecular perspective on signaling is still far from understood. The data presented in this study suggest that TJs might be one of these hubs, acting to suppress TGF-β signaling.

4 CONCLUSION

As we are rapidly increasing our knowledge of how tumors grow, turn invasive, and eventually metastasize to distant organs, we understand new levels of complexity from a molecular to multicellular level, and we are exploring many new ways to treat cancer patients, beyond the initial way to target uncontrolled cell growth. Our first study implies that this exploration might be fruitful, as inhibition of HKII, involved in metabolism, lead to re-sensitization of cisplatin treatment and less proliferation of SKOV-3-R cells. In addition to this successful attempt, we also study the changeable features of SKOV-3-R cells, and found them to display EMT and CSC characteristics. Something we observed, but were unable to utilize as treatments.

Although this study aim to find alternative ways of treating ovarian cancer, it is obvious from our study, that we are repeatedly going back to evaluate the (same old) cancer hallmark of uncontrolled cell growth and proliferation. Perhaps, as we are evaluating new therapeutics based on profoundly different molecular and cell-functional backgrounds, we also need to reinvent the design of our studies on drugs, and have courage to expand the variety of readouts not only concerning tumor cell growth. Successful treatment targeting transition may not at all concern cell growth, but still prove valuable for clinical outcome in patients. The limitations in design of this study might also indicate that we currently know too little about cell transition in general and in particular lack ideas of how to apply it to cancer therapies.

In the following studies of this thesis we made an attempt to penetrate the topics of cell conversion and EMT, to elucidate molecular mechanisms behind TGF-β driven breast cancer progression and invasion. Our reasoning behind all these three studies were that transition of tumor cells, from epithelial to mesenchymal-like, is made possible when factors maintaining the initial epithelial phenotype are lost. In study II and III we investigate the role of the two transcription factors C/EBPβ and Foxp4, and in study IV the TJ and virus receptor CAR. All our studies pointed towards a role of these factors in determining breast cancer cells response to TGF-β.

In the C/EBPβ study we found that loss of C/EBPβ during EMT through miR-155 resulted in reduced expression of E-Cadherin and CAR. Interestingly, it is also known that Smad and LAP2 together induce G1 arrest through transcriptional activation of p15INK4b upon TGF-β1 treatment, suggesting, together with our results, that the processes of cell growth and transition are linked together. In study III, Foxp4 was shown to, as a transcriptional repressor of Snail1, have the capacity to inhibit TGF-β induced EMT. In addition, this study indicates that oncogenic RAS signaling might trigger loss of Foxp4 in breast tumors, again showing a connection between cell growth and EMT. Regarding transcriptional regulation, these two studies together with many others on transcriptional EMT regulators, reveal that transcription programs involving repressors and activators are needed to maintain phenotypes and that alterations due to stimuli such as TGF-β can potentiate changes in cells morphology and functionality, causing EMT.

At a larger scale these studies hint towards the fact that molecules are versatile and shared between signaling pathways, used in different cellular processes, and linked together. In study IV we also find that CAR, previously thought of as a downstream target of TGF-β1 induced EMT and functionally promoting cell adhesion, to have an extended role in cell signaling. In fact, CAR serves to maintain Pten functionality at TJs, thus regulating Akt signaling which is highly coupled to EMT, but also cell growth, and other cellular processes. In the three last studies we did not characterize breast cancer cells, but aimed to study EMT,

in a hypothesis driven manner. Interestingly this has led us to a point where we, to some degree, understand the basis of cellular transition, not as a separate ontology or category of cellular processes, but in a bigger context being integrated with many.

Based on this understanding, it might be possible that the transitory capacity of tumors can be used to e.g. reactivate growth inhibitory or apoptotic responses to currently used drugs, as a potential therapy for cancer. We might also attempt to use the changeable behavior to our advantage, by influencing the cellular fate of tumor cells. Potentially, this could be achieved through reactivation of factors similar to those being presented study II-IV, pushing cancer cells towards an epithelial cell fate, and preventing EMT. On the other hand, we know that many factors, both intracellular and extracellular, are involved in the induction of EMT, and that transition, in opposite to cell death, is not a terminal stage. Thus, it might prove difficult to first of all prevent EMT in cancer, and secondly to maintain suppression over a suspended period of time.

Potential differentiation therapies might therefore profoundly differ from traditional chemotherapy, where treatments cannot rapidly break processes e.g. to induce cell death, but instead needs to work together with existing genetic programs in the cancer cells to achieve beneficial outcomes such as less invasion and EMT. Although EMT of tumor cells towards an invasive stage is well integrated with most (by today) defined hallmarks of cancer, it seems nevertheless plausible that great therapeutic benefits from work on cellular transition and differentiation, will occur only if we go beyond these hallmarks and even the cancerous disease itself. Perhaps, work in accordance with genetic programs in a therapeutic purpose will prove to be lifelong treatments.

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