Molecular factors influencing epithelial-mesenchymal transition in breast cancer

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To my family

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ABSTRACT

Epithelial-mesenchymal transition (EMT) is a developmental process defined by loss of epithelial characteristics and acquisition of mesenchymal phenotype. EMT or similar processes are also implicated in carcinoma cell invasion and the progression of breast carcinoma to metastasis. In a cell model system for mammary carcinogenesis it has previously been shown that signaling from the oncogenic receptor tyrosine kinase c-erbB2 (HER2), frequently overexpressed in mammary cancers, induces EMT. In this system, c-erbB2-induced EMT was significantly delayed by high cell-density and cell-cell-dissociation occurred before downregulation of the epithelial adhesion molecule E-cadherin. Loss of E-cadherin expression is generally viewed as a fundamental event in EMT. This thesis shows that ectopic expression of E-cadherin concomitant with c-erbB2 signaling did not hinder the progression of EMT. E-cadherin expressed in mesenchymal cells had a weaker attachment to the cytoskeleton, implicating that rearrangement of the cytoskeleton is an important mechanism in EMT-associated cell-cell-dissociation. Expression of dominant negative E-cadherin weakened cell-cell adhesion but did not enable EMT at high cell-density. These finding indicate that loss of E-cadherin is a consequence rather than a cause of EMT and that density-dependent inhibition of EMT is not mediated by E-cadherin. The expression of the transcription factor nuclear factor I-C2 (NFI-C2) is lost during mammary tumor progression and NFI-C2 has been shown to counteract EMT by repressing the transcription factor Forkhead box F1 (FoxF1). FoxF1 induces EMT and invasiveness in breast cancer cells. In this thesis, Affymetrix microarray was used to find oppositely regulated targets of NFI-C2 and FoxF1. The extracellular matrix enzyme lysyl oxidase (LOX) was found to be negatively regulated by NFI-C2 and positively regulated by FoxF1 and responsible for the increased invasiveness caused by FoxF1 overexpression. A signaling pathway was identified where FoxF1-induced upregulation of LOX activated focal adhesion kinase, subsequently suppressing Smad2 activity. In parallel, overexpression of FoxF1 activated the p38 MAPK signaling pathway. These findings give new insights into the regulation of signaling pathways known to be important during breast tumor progression. Based on the findings that NFI-C2 is lost during breast tumor progression and suppresses EMT, the prognostic value of NFI-C2 in a mixed cohort of breast cancer patients was investigated. NFI-C2 was found to be a powerful prognostic marker associated with good prognosis in breast cancer.

Keywords: breast cancer, epithelial-mesenchymal transition, c-erbB2, E-cadherin, NFI-C2, FoxF1, LOX

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LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Nilsson GM**., Akhtar N., Kannius-Janson M., Baeckström D. Loss of E-cadherin expression is not a prerequisite for c-erbB2-induced epithelial-mesenchymal transition. *International Journal of Oncology*. 2014 Jul;45(1):82-94.

II. **Nilsson G**., Kannius-Janson M. Forkhead box F1 promotes breast cancer cell migration by upregulating lysyl oxidase and suppressing Smad2/3 signaling. *Manuscript*.

III. Nilsson J., **Nilsson G.,** Nemes S., Kovács A., Helou K., Jirström K., Kannius-Janson M. Nuclear factor I-C2 is a powerful prognostic marker in breast cancer. *Manuscript*.

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ABBREVIATIONS

BCSS	breast cancer-specific survival
CDH-1	cadherin 1
CEL	carboxyl ester lipase
CSC	cancer stem cell
CTC	circulating tumor cell
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
FAK	focal adhesion kinase
Fox	forkhead box
Jak2	janus tyrosine kinase 2
kDa	kilodalton
LOX	lysyl oxidase
MAPK	mitogen activated protein kinase
MET	mesenchymal-epithelial transition
miRNA	micro-RNA
MMPs	matrix metalloproteinases
NFI	nuclear factor 1
NGF	nerve growth factor
PDGF	platelet-derived growth factor
PR	progesterone receptor
RFS	recurrence-free survival
RTK	receptor tyrosine kinase
TGF-β	transforming growth factor β
WAP	whey acidic protein

INTRODUCTION

Mammary gland

The mammary gland is a secretory organ, producing milk. It consists of a ductal network surrounded by a stroma compartment. Mammary ducts are formed by two main types of epithelial cells; luminal and basal. The luminal cells forms the ducts and alveoli and have secretory properties. The basal epithelium consists of contractile myoepithelial cells that surround the luminal epithelium. A basement membrane is lining the basal epithelium. The stroma compartment contains extracellular matrix (ECM), stromal cells, immune cells and adipocytes, making up the fat pad (Fig. 1) [1, 2].

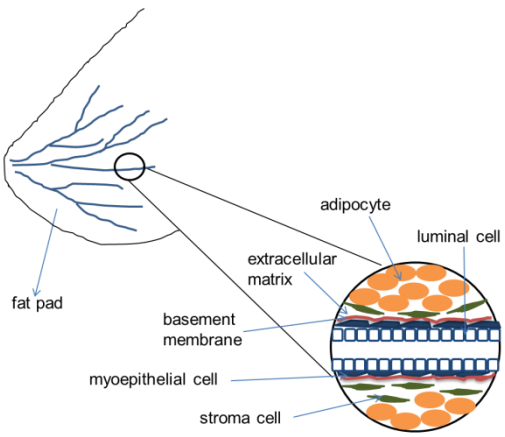

Figure 1. Schematic picture of the mammary gland

The development of the human breast is a progressive process proceeding in distinct phases; embryonic development, pre-pubertal growth, pubertal expansion, pregnancy- and lactation-associated remodeling, and post-lactational and post-menopausal involution [3]. The mammary gland reaches its full mature state during the pregnancy-lactation cycle. During the distinct phases of mammary gland development there is progressive ductal elongation and branching followed terminal differentiation at parturition and involution characterized by apoptosis and tissue remodeling during weaning [4, 5].

The development and function of the mammary gland are influenced by a number of growth factors and hormones, e.g. estrogen, progesterone, prolactin and epidermal growth factor [6, 7], and also dependent on

interactions between stromal and epithelial cells. Signals from stromal cells, as well as the composition of the ECM (laminin, fibronectin, collagen, proteoglycans etc.) help epithelial cells to generate and maintain apico-basal polarity [8]. In response to extracellular signals, integrins (transmembrane receptors for ECM) activate growth and survival signals [9]. Moreover, many of the factors important for mammary gland development are also implicated in breast cancer. During the development of the mammary gland the epithelium shows high levels of proliferation, reduced apico-basal polarity and epithelial-mesenchymal plasticity, characteristics also associated with tumorigenesis [10].

Breast cancer

Breast cancer development is a multi-step process categorized by different stages [11]. These stages include cellular immortality, hyperplasia, tumorigenicity and invasiveness. During these stages there is an initial epithelial hyperplasia, which can develop into atypical hyperplasia, *in situ* carcinoma, invasive carcinoma and metastasis. Breast cancer can begin in different areas of the mammary epithelium; the ducts or the lobules and most commonly derived from the luminal cells [12]. It is a heterogeneous disease with different subtypes. These subtypes show differences in the molecular profile, response to therapy and prognosis. The molecular subtypes are; luminal A, luminal B, molecular apocrine, basal-like, HER2, normal breast-like and claudin-low [13-15].

The mechanisms important for normal breast function are also important in suppressing the development of tumors by regulating proliferation and survival. Genetic and epigenetic changes leading to dysregulation of these normal processes can cause uncontrolled proliferation and imbalance between proliferation and apoptosis, promoting carcinogenesis. Inherited or acquired mutations in oncogenes (e.g. *HER2*, *MYC*, *CCND1*, *PIK3CA* [16-19]) and tumor suppressors (e.g. *P53*, *BRCA1/2*, *RB1*, *PTEN* [20-24]) are associated with breast cancer. Hormones and growth factors controlling normal mammary gland development together with their receptors and co-regulators are also involved in the development of breast cancer. If these factors are dysregulated it can lead to amplification of growth and survival signals [25].
In addition, microRNAs (miRNAs) have been shown to play key roles in carcinogenesis. miRNAs are small non-coding RNA molecules that control post-transcriptional gene expression by binding to target messenger RNAs, causing their degradation and downregulation of the target protein. Some function as tumor suppressors and others act as oncogenes [26-28].

As a response to the genetic alterations in the epithelial cells, there are alterations in the microenvironment, such as increased levels of growth factors and cytokines, and increased matrix synthesis, cross-linking and ECM stiffening. Matrix stiffening increases integrin signaling, promoting cell growth, survival and tumor progression [29-32].

It has been suggested that different cell types or transformation of mammary stem cells at different developmental stages are the cause of the different breast cancer subtypes. The more aggressive breast cancer subtypes are suggested to originate from mammary stem cells and the less aggressive subtypes from more differentiated mammary cells [2, 33-35].
Cancer stem cells (CSCs) were first identified in hematopoietic cancers and later in solid tumors such as breast tumors [36-38]. The existence of CSCs in the breast was suggested in a study demonstrating that only a minority of cancer cells have the ability to form new tumors. Tumorigenic (cancer-initiating) and non-tumorigenic cancer cells were identified based on differences in molecular markers [38]. Tumorigenic cancer cells have the ability to self-renew and produce progeny that are able to differentiate, which are properties of normal stem cells [39]. CSCs also have metastatic properties such as increased motility [40].

The invasion-metastasis cascade

Metastases are formed by a multi-step process. Cancer cells that have acquired the ability to disseminate from the primary tumor can travel through the blood and lymphatic system to distant sites in the body where they can form new colonies (metastases). The process when carcinoma cells acquire the ability to break through the basement membrane and invade the nearby stroma is termed localized invasion. The step when cancer cells enter the blood and lymphatic vessels is called intravasation. The migrating cells may die from anoikis, a form of apoptosis triggered by detachment from the ECM. They can also survive for long periods and in some instances extravasate into the surrounding tissue, leading to formation of micrometastases. If the foreign tissue microenvironment is favorable, the cancer cells may begin to proliferate and form a secondary tumor, a process termed colonization (Fig. 2) [41]. Carcinomas are benign as long as the tumor cells do not break through the basement membrane and malign when they have acquired the ability to metastasize which is the leading cause of death in cancer patients.

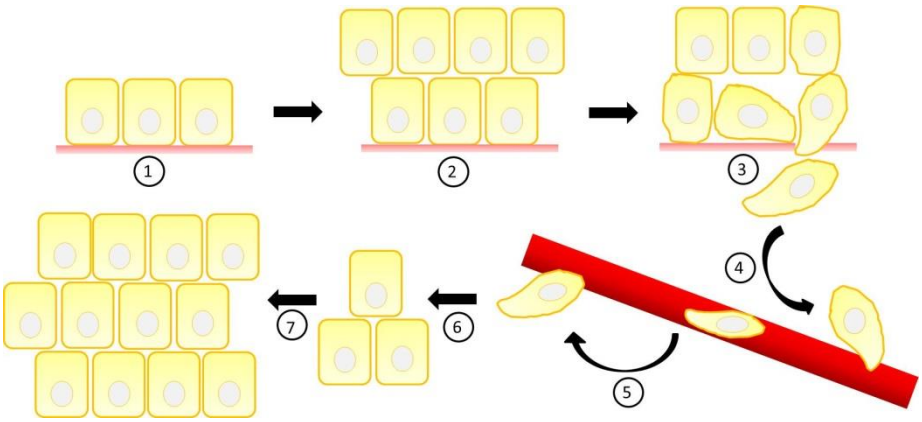

Figure 2. Schematic picture of cancer cell invasion and metastasis. 1. Normal epithelium. 2. Carcinoma in situ*, 3. Invasive carcinoma. 4. Intravasation. 5. Extravasation. 6. Micrometastasis. 7. Colonization.*

Epithelial-mesenchymal transition

Epithelial and mesenchymal cell phenotypes are not always permanent. During development it is critical that cells can change in morphology and behavior for proper morphogenesis. They do this by converting between the epithelial and mesenchymal phenotypes. These processes are termed epithelial-mesenchymal transition (EMT) and mesenchymal-epithelial transition (MET) [42]. EMT and MET are critical during developmental stages such as gastrulation [43], neural crest formation [44], and heart valve formation [45], as well as other morphogenetic events. EMT has also been observed during branching morphogenesis in the mammary gland [46].

EMT involves activation of genes that are critical for creating the mechanisms needed for cell migration through the ECM. Epithelial cells are attached to each other by different junctions (tight junctions, adherens junctions and desmosomes). During EMT, these junctions dissolve, allowing epithelial cells to separate, lose apical- basal polarity and gain motility. The epithelial cell-cell adhesion molecule E-cadherin, and epithelial-specific integrins, are replaced by mesenchymal specific N-cadherin and integrins specific for a more transient adhesion. The epithelial intermediate filaments, cytokeratins, are replaced by vimentin [47]. The underlying basement membrane is degraded by matrix metalloproteinases (MMPs) and the cell migrates into the surrounding stroma (Fig. 3). After migration to a distant site, the mesenchymal cells may return to a epithelial phenotype by undergoing MET [48].

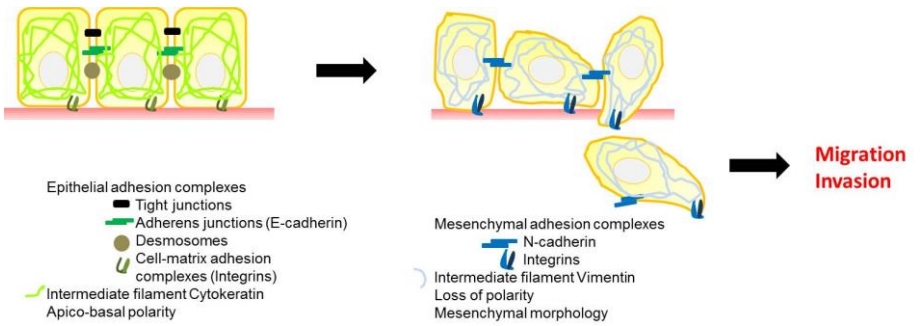

Figure 3. Epithelial-mesenchymal transition. To migrate and to invade a cell needs to detach from the neighboring cells in the epithelium. E-cadherin and epithelial adhesion complexes are replaced by mesenchymal adhesion complexes. Epithelial cells lose their polarity and gain motility and a mesenchymal morphology. The cytoskeleton is remodeled and vimentin is expressed. There is an increase in expression and secretion of extracellular components, e.g. fibronectin. The figure is adapted from [49].

Besides being critical during development, EMT is involved during wound healing. Keratinocytes at the edge of a wound undergo EMT and MET during the wound healing process [50]. EMT or EMT-like events have also been implicated in fibrosis [51] and cancer metastasis [52]. There are similarities, but also differences, between EMT events during development and other processes, leading to the classification of three different EMT types. Type 1 refers to developmental EMTs; type 2 includes EMT events in wound healing and fibrosis; and type 3 refers to the EMT associated with cancer metastasis. One difference between type 2 and 3 to type 1 is the existence of an inflammatory response, which does not exist in embryos [53].

Molecular players involved in EMT

EMT is driven by a network of transcription factors including Snail1, Snail2 (also known as Slug), and Snail3, Zeb1 and Zeb2, Twist1 and Twist2 and others. Their expression is triggered in response to extracellular signals derived from the cellular microenvironment. Potent inducers of EMT are members of the transforming growth factor–β (TGF-β) family [54, 55], epidermal growth factor (EGF) [56, 57], fibroblast growth factor (FGF) [58, 59] and platelet-derived growth factor (PDGF), but also Wnt [60], Notch [61], Hedgehog [62] and nuclear factor-κB (NF-κB) [63]. Receptor tyrosin kinases (RTKs), as well as other types of receptors, and a wide range of signaling pathways transduce EMT initiating signals and several pathways work in cooperation in order to full transition to a mesenchymal phenotype. Activation of Ras and Src pathways are involved in dissociation of adherens

junctions and desmosomes and remodeling of cytoskeleton by regulating the Rho family of GTPases [64]. Hypoxia (oxygen deprivation in the microenvironment) can also drive EMT during development or tumor progression. The hypoxic response is mainly mediated by hypoxia inducible factor-1 (HIF-1) which can promote EMT by regulation of Twist [65]. miRNAs have also emerged as key players in EMT and MET. The miRNA-200 family are proposed to be involved in reversible switching between epithelial and mesenchymal state [66].

EMT in cancer

In cancer, genetic and epigenetic events lead to alterations in cancer cell phenotype. These changes become most evident when cancer cells leave the epithelium and initiate metastasis (Fig. 2 and 3). Carcinoma cells at the invasive front of a tumor may lose epithelial traits and acquire a mesenchymal phenotype. Signals supplied by stromal cells, that secrete growth factors and cytokines, also contributes in the transition to mesenchymal phenotype [30, 67].
Signaling pathways and downstream effectors involved in the phenotypic changes needed for a carcinoma cell to detach from the epithelium seems to be highly similar to the events that orchestrate developmental EMT. Therefore, EMT or EMT-like events have been implicated in tumor invasion and metastasis, especially in the early steps of metastasis by facilitating cell dissemination and thereby generation of circulating tumor cells (CTCs). The revers process, MET, is believed to be responsible for the formation of metastases at distant organs [68]. Constitutive expression of transcription factors associated with EMT, suppresses metastasis formation, suggesting that EMT transcription factors must be downregulated for metastases to form [69-71]. In addition, high expression of E-cadherin is seen in breast cancer metastases [72, 73], suggesting that re-expression of epithelial markers is necessary for formation of metastases.

It is difficult to study EMT in human tumors, since EMT is considered a transient event and a tumor specimen represents only one instant. However, EMT in cultured cells and animal models has been intensely studied. Multiple signaling pathways and often cooperation of different pathways has been shown to be involved in the initiation and progression of EMT in cancer cell models. Overexpression of RTKs, e.g. EGF receptors, or constitutive signaling from Ras, can drive EMT by activating Raf/MAPK- and PI3K pathways leading to expression of transcription factors like Snail, Slug Twist and Zeb1 [74-76]. There are many studies implicating TGF-β as a master

regulator of EMT, and cooperation of TGF-β with Ras or RTKs has been shown to cause EMT [77].
Expression of mesenchymal markers have been observed in breast carcinomas of the basal and triple-negative subtypes [78]. However, such tumors also contain epithelial markers, suggesting that they have undergone a partial EMT [79, 80].
It has been proposed that EMT can generate CSCs [81], however the origin of CSCs is a subject of debate. It has been shown that in mammary epithelial cells, expression of Twist1 or Snail, or treatment with TGF-β increases the number of cells with CSCs properties ($CD44^{high}/CD24^{low}$) [82, 83]. EMT markers and stem cell markers are coexpressed in CTCs from breast cancer patients with metastasis [84, 85] .

Important factors controlling EMT

As described above there are many factors involved in EMT. The studies included in this thesis have focused specifically on four different factors that have been shown to be involved in breast tumor development and EMT. The following sections in the introduction will be about these factors.

E-cadherin

E-cadherin (E for epithelia), also known as cadherin-1 (CDH-1), uvomorulin and L-CAM, belongs to the type-1 classical cadherin family and is the major cell adhesion molecule (CAM) involved in binding between neighboring cells in adherens junctions.
Cadherins play a central role during the development and maintenance of multicellular organisms. E-cadherin helps to provide mechanically strong adhesive links between cells in the tissue and is important in defining apico–basal polarity and maintaining epithelial morphology. E-cadherin is expressed early in embryonic development and initiates polarization of cells to form the blastocyst [86]. E-cadherin knockout studies in mice show lethality at the blastocyst stage [87]. Conditional gene inactivation of E-cadherin in the mouse mammary gland leads to altered epithelial differentiation and cell death [88].

The extracellular domain of E-cadherin consists of five repetitive domains. The outermost part of E-cadherin contains an adhesion recognition motif important for binding specificity to identical cadherin molecules [89]. E-cadherin molecules form Ca^{2+} dependent complexes, also involving catenins; β-catenin/α-catenin or γ-catenin (plakoglobin)/α-catenin and p120 catenin, mediating linkage to the actin cytoskeleton (Fig. 4) [90-92]. β-catenin and γ-catenin both interact with E-cadherin but since their binding sites overlap

they cannot bind simultaneously. α-catenin is an actin binding protein and is believed to link the catenin-cadherin complex to the cytoskeleton [93]. However, it has been demonstrated that α-catenin cannot bind actin and β-catenin at the same time [94, 95], suggesting additional complexity.

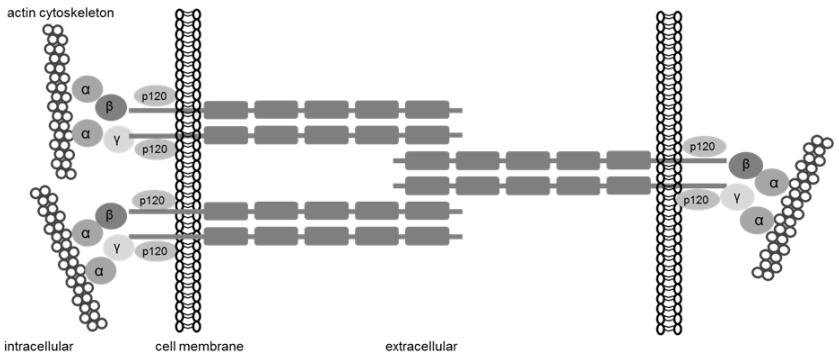

Figure 4. Schematic drawing of the organization of E-cadherin in adherens junctions. E-cadherin is a 120 kDa protein expressed on the surface of epithelial cells. E-cadherin contains five extracellular domains, a single transmembrane domain and a cytoplasmic domain which interacts with catenins connecting E-cadherin to the cytoskeleton; β-catenin (β), γ-catenin (γ) and α-catenin (α). p120 catenin binds to juxtamembrane domain of E-cadherin. E-cadherins exerts homophilic interactions between neighboring cells, forming a "zipper" structure.

Regulation of adherens junction formation and stability involves both transcriptional and posttranscriptional regulation of E-cadherin. The levels of E-cadherin expression [96] and the levels of cytosolic β-catenin regulate the strength of adhesion [97]. The amount of E-cadherin available for adhesion can also be regulated by recycling of endocytosed E-cadherin [98] and proteolytic cleavage [99].

E-cadherin expression is lost during the development of most epithelial cancers. Loss of E-cadherin expression is suggested to be one of the key steps in EMT, invasion and metastasis [100]. Consequently, E-cadherin is referred to as a tumor suppressor and forced re-expression of E-cadherin in carcinoma cells can reverse malignant properties [101]. Loss of E-cadherin expression is a typical feature of invasive lobular carcinoma [102]. However, other types of breast cancer show variable downregulation of E-cadherin expression [103, 104].

c-erbB2

The c-erbB family of RTKs, includes epidermal growth factor receptor (EGFR; c-erbB1, HER1), c-erbB2 (Neu, HER2), c-erbB3 (HER3) and c-erbB4 (HER4), which are activated by a number of ligands, e.g. EGF, TGF-α and heregulin. c-erbB receptors consist of an extracellular ligand binding domain, a single transmembrane domain and an intracellular kinase and substrate domain. Upon ligand binding they form a variety of homo- or heterodimers leading to activation and phosphorylation [105]. Activation of these receptors induces cell growth, proliferation, differentiation and survival signals, including Rho GTPases, the Ras-MAP kinase cascade, PI3K and phospholipase Cγ [106]. c-erbB2 and c-erbB3 activity require heterodimerization with other c-erbBs since c-erbB2 does not bind growth factor ligands, and c-erbB3 lacks intrinsic kinase activity [107]. The c-erbB family plays crucial roles in heart and nervous system development [108-111] and epithelial morphogenesis, for example in the development of embryonic and adult mammary gland [112-114].

c-erbB2 is a glycosylated protein with a molecular weight of 185 kDa. Amplification of c-erbB2 is a common feature in breast cancer. Overexpression is seen in 15-30 % of breast tumors and is associated with aggressive and invasive tumors and poor prognosis [16, 115]. Treatment with the monoclonal antibody trastuzumab (Herceptin) against c-erbB2 has a positive effect on overall survival and recurrence risk [116, 117]. Lapatinib, a tyrosine kinase inhibitor, is also used in treatment of c-erbB2 positive breast cancer [118]. Overexpression of the normal c-erbB2 protein, or point mutations in its transmembrane domain, has been shown to have a transforming effect on mammary epithelial cells. When overexpressed, c-erbB2 can form active homodimers in the absence of ligand [119-121]. Association between c-erbB2 homodimerization and gene amplification has been shown *in vivo*, which also was associated with poor prognosis [122]. c-erbB2 affects cell adhesion and migration, implicating a role for c-erbB2 in metastasis [123, 124]. Although the receptor commonly associated with EMT is the TGF-β receptor, there are studies also implicating c-erB2 in EMT. In an *in vitro* three-dimensional model system it was demonstrated that overexpressed c-erbB2 induces proliferation and disrupts tight junctions and epithelial polarity [125]. Overexpression of c-erbB2 in the mammary epithelial cell line MTSV1-7 inhibited transcription of E-cadherin [126]. *In vivo* selection of MCF-7 cells overexpressing c-erbB2 in nude mice, resulted in a sub-line that had underwent EMT [127].
CTCs expressing EMT- and stem cell-associated markers have been detected in the blood of patients with c-erbB2 positive metastatic breast cancer [128]. As mentioned, EMT has been proposed to generate CSCs [81]. CSCs often

exhibit resistance to therapy [129]. It has been suggested that the mechanisms leading to resistance to the c-erbB2-targeting drugs trastuzumab and lapatinib in c-erbB2 positive breast cancer may be linked to induction of EMT [130, 131].
In paper I, we have investigated the role of E-cadherin in c-erbB2-induced EMT.

Nuclear factor I-C2

Nuclear factor I-C2 (NFI-C2) is a member of the Nuclear factor I (NFI) transcription factor family. The NFI gene family consists of four members, NFI-A, NFI-B, NFI-C and NFI-X. NFI gene transcripts are differentially spliced [132] and expressed in unique but overlapping patterns [133-135]. The NFI proteins bind DNA as dimers at the consensus sequence TTGGC(N5)GCCAA and function as transcriptional activators as well as repressors of target genes [136]. NFI proteins are involved in regulation of genes controlled by e.g. insulin [137], TGF-β [138, 139], cAMP [137], steroid hormones [140], TNFα [141] and others. The unique expression patterns of NFI proteins suggests that they may play distinct roles in regulating tissue-specific gene expression during mammalian embryogenesis [134] and they have been demonstrated to be involved in cell differentiation [142, 143]. The oncogene Ras causes transformation of cells. It has been demonstrated that NFI can suppress Ras-induced transformation. Overexpression of NFI genes in chicken embryo inhibited transformation by subsequent overexpression of Ras in these cells [144]. Conversely, overexpression of Ras in mouse cells represses expression of NFI by affecting mRNA stability [145]. These studies suggest that loss of NFI function may promote tumor development. Furthermore, NFI proteins have been implicated in the function of the mammary gland after the identification of NFI binding sits in the genes for the milk proteins whey acidic protein (WAP) and carboxyl ester lipase (CEL) [146, 147].
Identification of specific NFI family members and isoforms involved in different processes has been hindered by the lack of specific antibodies. The responsible specific NFI protein is therefore unidentified in most studies.

Our laboratory has demonstrated that the specific isoform NFI-C2 is the protein responsible for activation of the WAP and CEL promoters in the mouse mammary gland at mid-pregnancy [148]. Further studies suggest a broader role for NFI-C2 in the mouse mammary gland. Besides activation of milk genes, it was shown that NFI-C2 activates the tumor suppressor gene p53 at mid-pregnancy [149]. From these studies it was suggested that NFI-C2 might participate both in the establishment of a functional gland and in the protection of the gland against tumorigenesis during proliferation.

The polypeptide hormone prolactin plays a critical role both in the differentiation of the gland during pregnancy and in the regulation of milk protein gene expression [150]. The prolactin receptor can activate a number of signaling pathways [151]. In mammary epithelial cells, prolactin binding to the prolactin receptor activates Jak2 (Janus tyrosine kinase 2). Activated Jak2 phosphorylates Stat5 (signal transducer and activator of transcription 5) that dimerizes and translocates to the nucleus where it binds and activates target genes [152]. The prolactin signaling pathway has also been demonstrated to play a role in maintenance of the NFI-C2 protein in mammary epithelial nuclei by regulating NFI-C2 protein levels. This occurs by a mechanism distinct from prolactin's regulation of Stat5 [153]. Further studies demonstrated that prolactin via nuclear Jak2 regulates the amount of active NF1-C2 through tyrosine phosphorylation and protection against proteasomal degradation (Fig. 5) [154].

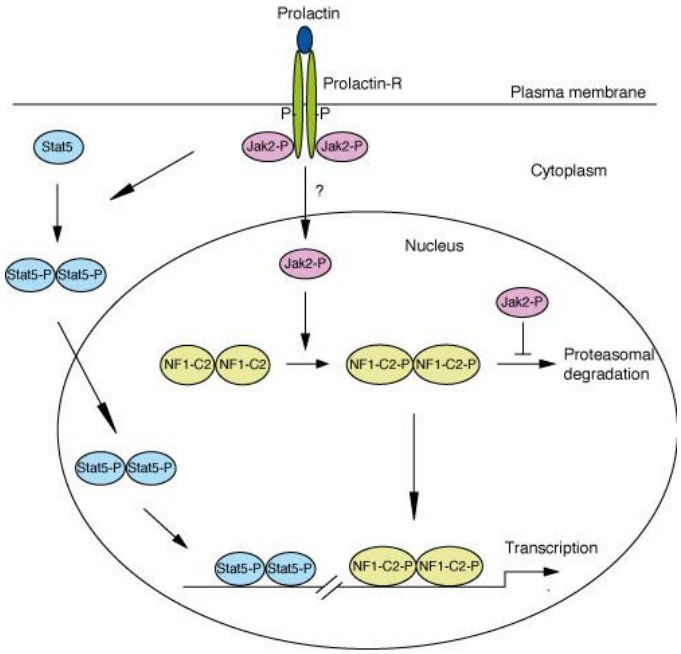

Figure 5. Proposed model of the prolactin-nuclear Jak2/NFI-C2 signaling pathway in mammary epithelial cells. Activation of Jak2 by prolactin/prolactin receptor (Prolactin-R) translocates Jak2 to the nucleus by an unknown mechanism. In the nucleus, Jak2 phosphorylates NF1-C2 on tyrosine(s) and protects it from proteasomal degradation. The classical way by which prolactin transduces its signal in mammary gland is through the Jak2-Stat5 pathway. The figure is reprinted with permission from Nilsson et al [154].

The prolactin-nuclear Jak2/NFI-C2 signaling pathway was later implicated in the regulation of tumor development and EMT. It was shown that patients

with nuclear NFI-C2 in their breast cancer cells have better prognosis compared to those without detectable NFI-C2, and that NFI-C2 suppresses invasiveness and EMT by repressing the transcription factor Forkhead box F1 (FoxF1) [155]. Paper II and paper III are continuations of that study, which will be discussed in more detail below.

Forkhead box F1

Forkhead box (Fox) proteins constitute a family of transcription factors that are related through the presence of a conserved forkhead or winged-helix DNA-binding domain. Fifty Fox genes have been identified in the human genome (FoxA to FoxS). Fox proteins bind DNA at the recognition core motif 5' ((G/A)(T/C)(C/A)AA(C/T)A) 3'. Nucleotides outside this core motif are important for the specificity between the different Fox proteins [156]. Temporal and spatial restriction of expression patterns provides an additional level of specificity, as well as context dependence. For example, in the developing lung, FoxF1 and FoxF2 have overlapping but not identical expression patterns, suggesting that these transcription factors may have both similar and independent functions [157, 158].

FoxF1 acts as a transcriptional activator or repressor of target genes [157, 159]. Early in development FoxF1 is required for differentiation of extra-embryonic and lateral plate mesoderm and for mesodermal proliferation in the primitive streak. Inactivation of FoxF1 in mouse embryos is lethal and the embryos have abnormalities in the coelom, the amnion fails to expand and there is a lack of vasculogenesis [160]. Later in development, FoxF1 is expressed in the mesenchyme adjacent to the epithelium in the alimentary, respiratory and urinary tracts, where it is believed to regulate mesenchymal-epithelial interactions [157]. In the developing lung, FoxF1 transcription is activated by Sonic hedgehog signaling [161, 162]. The effects of FoxF1 have been shown to be dependent on gene dosage [163]. FoxF1 heterozygous mouse pups show developmental defects in the gut, lungs, trachea, esophagus and gallbladder [161, 163-165]. In the adult, expression of FoxF1 is found in a range of tissues, e.g. in the gut, brain, eye and lung [166, 167]. In humans, the neonatal lethal disorder Alveolar capillary dysplasia with misalignment of pulmonary veins (ACDMPV) is linked to inactivating mutation of FoxF1 [168].

Fox proteins are important in a variety of biological processes, including metabolism, development, differentiation, proliferation, apoptosis and migration. As a consequence, loss or gain of Fox function can lead to tumorigenesis. Fox proteins have been suggested to act as either tumor suppressors or oncogenes [169].

The first study demonstrating a role of FoxF1 in tumorigenesis came from our laboratory. There, it was shown that in a mouse mammary epithelial cell line, FoxF1 overexpression induced EMT, migration and invasiveness. In addition, it was demonstrated that forced expression of FoxF1 enhanced xenograft tumorigenesis in nude mice [155]. FoxF1 has been shown to be important for mesenchymal cell migration by directly repressing integrin β_3 expression [159]. FoxF1 has also been shown to play a role in lung cancer associated fibroblasts, where FoxF1 expression stimulated cell migration and xenografted tumor growth [170]. In paper II, we have investigated the mechanisms responsible for FoxF1-enhanced invasion.

AIMS

The objective of this thesis was to study the influence of different molecular factors on the EMT process associated with breast cancer cell invasion and metastasis and their possible use as biomarkers.

The aim of paper I was to study the influence of oncogenic tyrosine kinase receptor signaling on EMT; specifically, to investigate the role of E-cadherin during c-erbB2-induced EMT, and in high cell-density dependent inhibition of EMT.

In paper II, the objective was to study the role of NFI-C2 and FoxF1 in mammary carcinogenesis by identifying factors regulated by these transcription factors and involved in EMT and invasion and elucidate what mechanisms are responsible for FoxF1-enhanced invasion.

Paper III aimed to investigate the prognostic value of NFI-C2 in breast cancer.

SPECIFIC BACKGROUND

Paper I

The human mammary epithelial cell line HB2 [171] is a subclone from the non-tumorigenic MTSV1-7 cell line. MTSV1-7 cells were obtained from luminal epithelial cells from milk and immortalized with the SV40 T antigen [172]. HB2 cells form spherical colonies in collagen and has been used for *in vitro* studies of branching morphogenesis [171]. Constitutive overexpression of c-erbB2 in MTSV1-7 cells causes irreversible changes in morphology and repression of E-cadherin [126, 173]. To study the order of events in c-erbB2-signlaing effects, an inducible system in MTSV1-7 cells and HB2 cells was developed by Baeckström *et al* [174], using the hybrid receptor construct trk-neu [175]. Trk-neu consists of the extracellular domain of the trkA nerve growth factor (NGF) receptor and the transmembrane and cytoplasmic domain of c-erbB2 (neu). c-erbB2 homodimerization and activation can be induced by NGF treatment of the cells (Fig. 6).

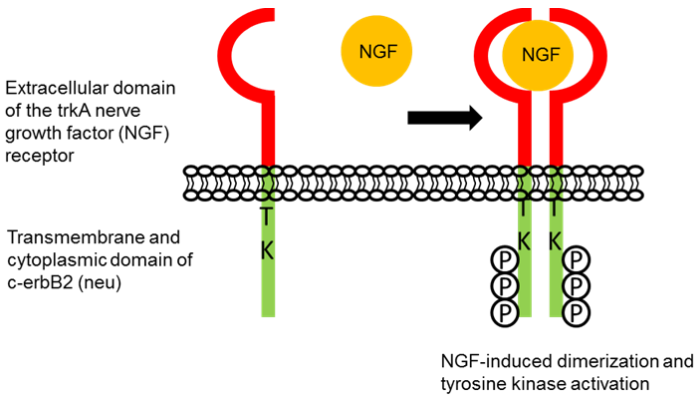

Figure 6. The trk-neu hybrid receptor. The extracellular domain of trkA nerve growth factor (NGF) is fused to the transmembrane and cytoplasmic domains of c-erbB2 (neu). NGF treatment induces homodimerization and tyrosine kinase (TK) activation and mimics c-erbB2 homodimerization.

Induced c-erbB2 signaling inactivated integrin $\alpha_2\beta_1$ leading to scattered growth in collagen. In addition, prolonged activation of c-erbB2 led to EMT [174]. Further studies using the high expressing clone HB2/tnz34 showed that c-erbB2-induced integrin inactivation was mediated by MEK/ERK signaling and PI3K dependent ILK and PKB signaling pathways [176]. Subsequent studies demonstrated that the EMT caused by prolonged induction of c-erbB2 signaling in HB2/tnz34 cells was irreversible and

associated with anchorage independent growth [177, 178]. Furthermore, c-erbB2-induced EMT was suppressed when cells were grown at high density. It was hypothesized that E-cadherin homophilic interaction can suppress EMT. It was also observed that the initial cell-scattering associated with EMT occurred before downregulation of E-cadherin [177].
In paper I, we aimed to analyze a possible role for E-cadherin in cell-cell contact-inhibition of c-erbB2-induced EMT.

Paper II

In paper II we used three different cell lines; HC11, MDA-MB-436 and HB2. HC11 cells are a mouse epithelial cell line cloned from the COMMA-1D cell line, which is derived from mammary tissue of BALB/c mice at mid-pregnancy [179, 180]. HC11 cells have been used for *in vitro* studies of epithelial cell proliferation, signal transduction and differentiation. MDA-MB-436 is a mesenchymal human breast carcinoma cell line derived from metastatic site (pleural effusion) and have been used for studying triple-negative breast cancer [181]. HB2 cells are described in the previous section.
As mentioned, in a previous paper it was demonstrated that the prolactin/nuclear Jak2/NFI-C2-pathway plays a role in breast cancer by regulating EMT, motility and invasion [154]. In order to study the effects of NFI-C2 without the involvement of other effectors stimulated by prolactin, a stable form of NFI-C2 (NFI-C2S) was stably transfected in HC11 cells. A point mutation protects NFI-C2S from proteasomal degradation in the absence of nuclear Jak2. NFI-C2S expression in HC11 cells increased epithelial characteristics and impeded migration. NFI-C2S expression in mesenchymal MDA-MB-436 cells lead to loss of some mesenchymal characteristics, decreased *in vitro* invasion and abolished xenografted tumor growth in nude mice, suggesting that NFI-C2 is a suppressor of tumor progression and EMT. This was underscored by the observation that NFI-C2 status of primary breast cancer correlated with survival and its expression was lost in lymph node metastases (discussed in the next section, paper III). Affymetrix microarray analysis of the transcriptome of MDA-MB-436 cells expressing NFI-C2S or control vector identified the *FoxF1* gene to be one of the most downregulated by NFI-C2, and it was also shown that *FoxF1* is a direct target of NFI-C2. FoxF1 overexpression in HC11 cells induced EMT, increased *in vitro* invasion capacity and enhanced xenografted tumorigenesis in nude mice.
In paper II, we wanted to further study the roles NFI-C2 and FoxF1 in EMT and invasion, primarily by identifying targets that are oppositely regulated by these factors and involved in EMT and invasion.

Paper III

Prognostic markers are used in the evaluation of a cancer patient's risk of relapse and disease progression. Prognostic markers include tumor size, axillary lymph node status, histologic grade, patient age and menopausal status. In addition, molecular markers are used to further help to predict prognosis or treatment response, e.g. Ki67, estrogen receptor (ER), progesterone receptor (PR) and HER2. However, these factors are unable to accurate predict which tumor will recur and to indicate responses to different therapies and therefore there is a need to find new prognostic markers.

It has previously been demonstrated that the presence of NFI-C2 in breast tumors has a clinical relevance [155]. The status of NFI-C2 was analyzed by immunohistochemistry in a tissue microarray containing 292 samples from patients diagnosed with stage II invasive breast cancer. Tumor cells had a weaker staining of NFI-C2 compared to normal glandular cells and only 1 of 159 lymph node metastases stained positive for NFI-C2. Patients with NFI-C2 in their tumor cells (74 of 292) had better prognosis compared to those without detectable NFI-C2, suggesting that NFI-C2 is a tumor suppressor. Furthermore, there was a correlation of nuclear Jak2 and NFI-C2 in tumor biopsies, indicating that the regulation of the nuclear levels of NFI-C2 by the prolactin/Jak2/NFI-C2 pathway also occurs in cancer.

These findings, together with the finding that NFI-C2 suppresses EMT, make NFI-C2 a candidate as a prognostic marker. In paper III, we aimed to investigate the prognostic value of NFI-C2 in a mixed cohort of breast cancer patients. We also analyzed the levels of NFI-C2 protein after tamoxifen treatment in cellular extracts from T47D and 4T1 cells. T47D is a human breast cancer cell line derived from metastatic site (pleural effusion). This cell line has epithelial morphology and is non-invasive. 4T1 is a mouse mammary tumor cell line with epithelial morphology. 4T1 cells form highly metastatic tumors when injected to BALB/c mice and are frequently used as a model for metastatic breast cancer. This is also a luciferase based model and the metastatic process can be followed by using bioluminescence in live animals.

RESULTS AND DISCUSSION

Loss of E-cadherin expression is not a prerequisite for c-erbB2-induced epithelial-mesenchymal transition (paper I).

In paper I, we have further investigated earlier observations that cells undergoing the first scattering phase in c-erbB2 induced EMT still expressed surface-bound E-cadherin, and that the progression of EMT was delayed by cell-cell contact [177]. These observations raised the questions whether loss of E-cadherin expression is a cause or a consequence of EMT and if cell density-dependent suppression of EMT is mediated by E-cadherin binding between cells.

In order to analyze a possible modulation of c-erbB2-induced EMT by E-cadherin, an inducible system for ectopic expression of E-cadherin in cells stably expressing the trk/neu hybrid receptor (HB2/tnz34) was created. This generated the clones TrE-ep1 and TrE-ep5 (ep for epithelial morphology). TrE-ep5 cells were grown at low density with a combination of c-erbB2 signaling and ectopic E-cadherin expression. By subsequently analyzing changes in expression and localization of epithelial- and mesenchymal markers, using flow cytometry and immunofluorescence, we could show that the forced expression of E-cadherin did not prevent the progression of EMT, as compared to cells with c-erbB2 signaling only. EMT requires loss of cell-cell adhesion and since E-cadherin is a major component in cell-cell contacts, loss of its expression is considered to be a fundamental event. However, it has been observed also in other model systems that EMT can occur without simultaneous downregulation of E-cadherin. For example, EGF-induced EMT in MDA-MB-468 cells occurred without complete downregulation of E-cadherin [182, 183]. In the present study, we showed that cell scattering following overnight EGF treatment of MDA-MB-468 cells occurred without major changes in surface-bound E-cadherin, implicating that downregulation of E-cadherin is not essential in at least the early phase of EMT.
In a fibroblastic clone (TrE-fib), isolated after prolonged c-erbB2 signaling in the presence of E-cadherin, the endogenous *CDH1* gene had been silenced. Induced expression of ectopic E-cadherin did not have any effect on morphology in these cells, suggesting that E-cadherin had lost its function. Performing dissociation assays on confluent TrE-ep5 and TrE-fib cells showed that induced expression of E-cadherin increased cell-cell adhesion in TrE-ep5 cells. In contrast, in fibroblastic TrE-fib cells, E-cadherin did not

have a restoring effect on cell-cell adhesion. These results further supported our assumption that the function of E-cadherin is lost in fibroblastic cells. In addition, the attachment of E-cadherin to the cytoskeleton was weaker in fibroblastic cells and β-catenin and α-catenin were localized to the cytoplasm and nucleus, although the localization of E-cadherin was grossly the same in epithelial and fibroblastic cells. It has previously been shown that c-erbB2 signaling in HB2 cells results in destabilization of the cortical cytoskeleton [184]. Rearrangements of the cytoskeleton may lead to difficulties in supporting E-cadherin mediated cell-cell adhesion, allowing cells to separate even in the presence of surface-bound E-cadherin.

The observation that the progression of c-erbB2-induced EMT was delayed by cell-cell contact raised the question whether cell-cell contact results in intracellular signaling events that hinder the EMT process. If such cell-cell contact-dependent signaling events are mediated by E-cadherin, then interfering with E-cadherin function would allow cells grown at high density to undergo c-erbB2-induced EMT. To test this hypothesis, we expressed a mutant E-cadherin, where the adhesive function is abolished [90]. Cells expressing this mutant construct, TrE^{wv}-ep, showed a decrease in cell-cell adhesion. However, the combination of c-erbB2 signaling and mutant E-cadherin expression in high density cultures did not result in progression of EMT. This indicates that E-cadherin is not involved in mediation of density-dependent inhibition of c-erbB2-induced EMT.
It has been demonstrated that MMP3 and Rac1b, factors that stimulate alterations in the cytoskeleton, induce EMT in mammary epithelial cells. This MMP3/Rac1b-induced EMT was dependent on the cells ability to spread over a larger surface. When the available surface area was limited MMP3/Rac1b-induced EMT was blocked [185], suggesting cell shape as an important factor in the initiation of EMT.
Our findings suggest that loss of E-cadherin expression may be a consequence rather than a cause of c-erbB2-induced EMT, and further that density-dependent inhibition of c-erbB2-induced EMT is not mediated by E-cadherin. We propose that cytoskeletal rearrangements in the initial phase of EMT may be the mechanism leading to cell-cell separation by impairment of E-cadherin function and cell-cell adhesion. These rearrangements might also be important in density-dependent inhibition of EMT by eliciting signals, in crowded cells, that control EMT progression.

Forkhead Box F1 promotes breast cancer cell migration by upregulating lysyl oxidase and suppressing Smad2/3 signaling (paper II).

The aim of this study was to further investigate the roles of NFI-C2 and FoxF1 in EMT and invasion. Using Affymetrix microarray analysis, we could compare the transcriptome of HC11 wild type cells to that of cells overexpressing NFI-C2S or FoxF1 and find targets that are oppositely regulated by these factors, involved in EMT and invasion. In order to identify factors that might be generally important in EMT and regulated by NFI-C2, we used the data from a previous array where the transcriptome of MDA-MB-436 cells was compared to that of MDA-MB-436 cells overexpressing NFI-C2S [155]. 45 genes were downregulated 1.5 fold or more by NFI-C2 in both these cell lines. Of these 45 genes, 17 were upregulated 1.5 fold or more by FoxF1 overexpression in HC11 cells. The gene that was by far most upregulated by FoxF1 was lysyl oxidase (*LOX*). LOX is an ECM enzyme that catalyzes the cross-linking of collagens or elastin, thereby controlling the structure and tensile strength of the ECM, but is also implicated in EMT and tumor progression to metastasis [186-189]. LOX has been demonstrated to be upregulated in invasive breast cancer cell lines and breast carcinomas [190, 191]. LOX was therefore a potential factor involved in the enhanced *in vitro* invasion capacity observed following FoxF1 overexpression in HC11 cells. We could detect high levels of secreted LOX in culture media from HC11FoxF1 cells. In addition, we could show that the enhanced *in vitro* invasion capacity of HC11FoxF1 cells was mediated by LOX, as treatment with a LOX inhibitor or reduction of LOX expression using RNAi, significantly decreased the capacity of HC11FoxF1 cells to migrate through matrigel. Taken together, we found LOX to be downregulated by NFI-C2 and upregulated by FoxF1 and responsible for the increased invasion capacity of HC11FoxF1 cells. These findings together with the previous observations that FoxF1 is repressed by NFI-C2 which expression is lost during tumor progression, suggests that loss of NFI-C2 may lead to expression of factors like FoxF1 and LOX leading to EMT and carcinoma cell dissemination.

Several of the 17 genes that were shown to be downregulated by NFI-C2 and upregulated by FoxF1 are known to be involved in TGF-β signaling and TGF-β associated EMT. In addition, LOX has been shown to be involved in TGF-β induced EMT [192]. This raised the question whether FoxF1 is involved in the regulation of TGF-β signaling pathways.
TGF-β is important for mammary gland development and function and in the suppression of tumorigenesis. Breast cancer progression can convert the function of TGF-β to become a tumor promoting factor [193, 194]. The

oncogenic activity of TGF-β is suggested to be a result from imbalance between canonical (Smad2/3) and non-canonical (non-Smad) TGF-β signaling systems, where the non-canonical pathways are believed to promote EMT and invasive and metastatic properties of cancer cells. The non-Smad pathways include various branches of MAP kinase pathways, e.g. p38 MAPK [195].
We investigated whether TGF-β signaling pathways are affected by FoxF1 and found that the Smad2/3 pathway is suppressed while the p38 MAPK signaling pathway is activated in HC11FoxF1 cells.

LOX has been demonstrated to control the activity of focal adhesion kinase (FAK) [32, 186, 189, 196]. We found that FoxF1 activates FAK in a LOX-dependent manner, as depletion of LOX by RNAi decreased the activity of FAK. In addition, LOX depletion activated the Smad2/3 signaling pathway by increasing the levels of Smad2/3 and the phosphorylation of Smad2. However, FAK inhibitor treatment showed that FAK is involved in the activation of Smad2 but not in the regulation of total Smad2/3 levels, suggesting that downstream of LOX there are additional factors involved in the regulation of Smad2/3 levels. The main findings are summarized in figure 7.

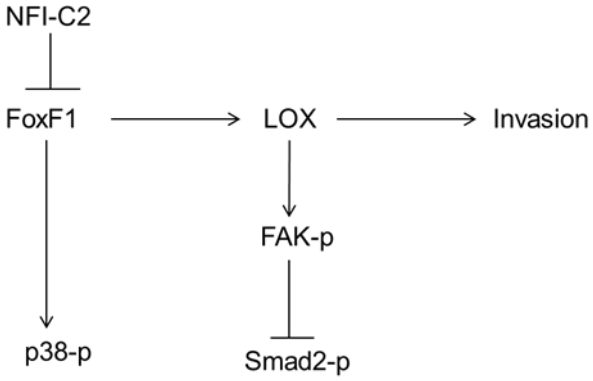

Figure 7. Schematic picture of signaling pathways regulated by FoxF1.

In this study we found LOX, which is upregulated in breast carcinomas and shown to promote metastasis, to be highly expressed in response to FoxF1 overexpression. In addition, FoxF1-induced invasiveness was shown to be dependent on LOX activity, suggesting that induction of EMT and invasiveness by FoxF1 may have an important role during breast tumor progression to metastasis. Furthermore, we found FoxF1 to be involved in TGF-β signaling pathways known to be important during breast cancer progression. However, the possible connection between FoxF1 and TGF-β

and whether these factors cooperate to influence metastatic activity needs to be further investigated.

Nuclear factor I-C2 is a powerful prognostic marker in breast cancer (paper III).

In this study, a tissue microarray was constructed from the tissue samples of 498 patients with primary invasive breast cancer diagnosed at the Malmö University Hospital, Malmö, Sweden, between 1988 and 1992 [197]. Of these 498 patients the expression level of NFI-C2 could be analyzed in 462 cases, using immunohistochemistry. 217 patients stained positive and 245 stained negative for NFI-C2. The expression of NFI-C2 was positively associated with advanced age, ERα expression and PR expression, and negatively associated with HER2 overexpression, lymph node status, tumor size, Ki67 expression and histological grade. This shows that NFI-C2 is positively associated with markers for good prognosis, suggesting that tumors with NFI-C2 expression may be less aggressive than tumors without NFI-C2 expression.
Kaplan-Meier analysis of all patients showed a significantly lower risk for recurrence and mortality from breast cancer for NFI-C2 positive compared to NFI-C2 negative patients, demonstrating an association between NFI-C2 and good prognosis. However, this association was only significant for patients with ERα-positive tumors.

Kaplan-Meier analysis of the patients that had not received any adjuvant treatment showed that NFI-C2 was associated with increased recurrence-free survival (RFS) and breast cancer-specific survival (BCSS), demonstrating a prognostic value of NFI-C2. Multivariate Cox analysis including age, tumor size, ERα, PR, Ki67, HER2, and lymph node status demonstrated that NFI-C2 was an independent predictor of RFS. NFI-C2 remained an independent predictor of RFS in the ERα-positive group, indicating that NFI-C2 has an additional value in predicting breast cancer recurrence besides that of the ER status.
NFI-C2 suppresses EMT and its expression is lost during breast cancer progression [155], suggesting that loss of NFI-C2 may facilitate the dissemination of carcinoma cells from the primary tumor and thereby the generation of CTCs. It has been demonstrated that the presence of CTCs correlates with lymph node metastases [198]. We therefore investigated the association between NFI-C2, lymph node status and RFS in the group of patients that had received tamoxifen treatment. The group of untreated patients could not be analyzed due to the low number with multiple lymph node metastases. We found that NFI-C2 was positively associated to RFS in

patients with no lymph node metastases whereas in patients with multiple lymph node metastases, NFI-C2 was negatively associated to RFS. Furthermore, lymph node status was only prognostic in the group of patients with NFI-C2 positive tumors. These data suggest that the role of NFI-C2 changes during breast cancer progression.
We investigated whether tamoxifen had an effect on NFI-C2 protein levels in two different breast cancer cell lines and found that tamoxifen reduced the levels of NFI-C2 in 4T1 cells, known to form metastases, while it had no effect on NFI-C2 levels in the non-invasive T47D cell line. Removing tamoxifen from 4T1 cells restored the levels of NFI-C2.

This study supports our hypothesis that NFI-C2 is a tumor suppressor and shows that NFI-C2 is a prognostic factor associated with good prognosis in breast cancer. However, it also provides data suggesting that the presence of NFI-C2 may in some instances instead be negative. NFI-C2 protein levels were differently affected by tamoxifen which might be dependent on the cellular phenotype. NFI-C2 has been shown to promote MET [155], which is believed to be a critical process in metastasis formation (described in the introduction). In patients with no lymph node metastases the presence of NFI-C2 would have a positive effect inhibiting EMT to occur. However, in patients with many lymph node metastases and presumably also harboring CTCs in their blood and lymphatic system, the presence of NFI-C2 in these circulating cancer cells might lead to MET and an increased risk for colonization and metastases formation. Our results indicate that during tamoxifen treatment the NFI-C2 levels are low whereas they increase when treatment is ended. If patients who have ended a tamoxifen treatment still has CTCs and the levels of NFI-C2 would increase, this might lead to a higher risk for metastases formation.

FUTURE ASPECTS

The subject of this thesis was to investigate how different molecular factors influence EMT. This thesis shows that c-erb2-induced EMT can proceed in the presence of the adhesion molecule E-cadherin and that cell density-dependent inhibition of EMT is not mediated by E-cadherin. Furthermore, it demonstrates an involvement of NFI-C2 and FoxF1 in the regulation of molecular factors and signaling pathways that are associated to EMT and invasion. We identified a signaling pathway where FoxF1 upregulated LOX in a FAK-dependent manner leading to suppression of Smad2/3 and increased invasion. In addition, NFI-C2 was confirmed to be a prognostic marker associated with good prognosis in breast cancer. In future work there are several aspects that can be further investigated.

The occurrence of a mechanism that can hinder EMT is intriguing. If EMT events are the cause of carcinoma cell dissemination and invasion, understanding such a mechanism would give critical insights into the process of metastases and possibly identify potential therapy targets. The effects of cytoskeletal rearrangements and cell shape seem to be important for the EMT process to occur and would be interesting to investigate further. Identifying factors that induce cytoskeletal rearrangements and cell spreading (including cell-surface targets other than E-cadherin) during EMT initiation would give information about a possible association between cell morphology and carcinoma progression.

To get further insight into the role of FoxF1 during carcinogenesis it would be of importance to evaluate the effects of FoxF1 *in vivo*. In an ongoing study, a transgenic mouse model for mammary carcinogenesis is used where c-myc is specifically overexpressed in the mammary epithelium under control of the WAP-promoter, causing tumor development after pregnancy [199]. In this model, FoxF1 is specifically knocked-out in the epithelium by utilizing the cre/lox system where cre is expressed under the control of the promoter of keratin 14. This model system will give insights into the effects of FoxF1-removal on tumor development and progression.
In addition, the 4T1 cell line can be used to study FoxF1 effect on metastatic capability. We hypothesize that implantation of 4T1 cells overexpressing FoxF1 into the fat pads of BALB/c mice may result in less formation of metastases compared to control. FoxF1 overexpression would possibly result in cancer cells with mesenchymal properties which would impede the ability to colonize and form metastases.

The role of NFI-C2 as a prognostic marker was evaluated in a nonrandomized trial. In order to evaluate the treatment predictive value of NFI-C2, a randomized trial needs to be used containing a large number of patients that has or has not received treatment.

To approach the question whether the level of NFI-C2 protein affects the tumor cells' capacity to metastasize, the 4T1 cell line can be utilized. 4T1 cells where NFI-C2 has been knocked-down and control cells can be implanted into the fat pads of BALB/c mice and their metastatic capability compared. If NFI-C2 promotes MET then the NFI-C2 knock-down cells will be more mesenchymal, and as a consequence of that, less capable to colonize and form metastases.
To elucidate the dual effects of NFI-C2 positivity depending on tumor progression stage, one can overexpress NF1-C2 in 4T1 cells. Implantation of these cells in the mammary fat pads of BALB/c mice might give rise to tumors with less metastatic capability compared to control. On the other hand, if NFI-C2 overexpressing cells instead were injected into the tail, resulting in cells entering the bloodstream, this might instead increase the metastatic capability. We hypothesize that NFI-C2 overexpression would generate cancer cells with epithelial properties enabling attachment at distant sites and metastasis formation.

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