New players, old suspects

and a novel line of action in EMT

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Chantal Heck

aus Buochs/NW

Basel, 2012

Genehmigt von der Philosophisch - Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Gerhard Christofori

Prof. Dr. Markus Affolter

Basel, den 13. Dezember 2011

Prof. Dr. Martin Spiess

Dekan

SUMMARY

Cancer is one of the most fatal diseases in humans, accounting for 13 % of all deaths worldwide through the destruction of healthy tissue and organs by uncontrolled proliferation. The most life-threatening step during tumor progression is reached when cancer cells leave the primary tumor mass to invade the surrounding tissue and colonize distant organs. This process of metastasis includes dissemination throughout the body by entering the blood - or lymphatic system to reach distant organs and form secondary tumors. To infiltrate the surrounding tissue, single motile tumor cells leave the tumor mass by breaking down cell-cell contacts, in a process called epithelial to mesenchymal transition (EMT). EMT is a complex molecular and cellular program enabling epithelial cells to abandon their differentiated phenotype, including cell-cell adhesion and cell polarity, and to acquire mesenchymal features and invasive properties.

One of the most prominent inducers of EMT is transforming growth factor β (TGFβ), which can suppress tumor formation by growth inhibition but also promotes tumor progression and metastasis in advanced tumors. To dissect the molecular mechanism underlying the complex process of EMT and understand the dual role of TGFβ during cancer progression we established different *in vitro* EMT model systems to identify a global EMT gene expression signature. By analyzing the commonly altered gene expression profiles we identified the transcription factor forkhead box protein F2 (Foxf2), neural cadherin (N-cadherin) and various cancer stem cell associated genes, to be upregulated during the EMT process.

Our study revealed that Foxf2 upregulation, although not required to gain mesenchymal markers, is essential for the disruption of cell junctions and the downregulation of epithelial markers in NMuMG cells treated with TGFβ. We show that during EMT, Foxf2 regulates the expression of the E-cadherin mediators zinc finger E-box binding homeobox 1 and 2 (ZEB1 and ZEB2) and the inhibitor of differentiation 2 (Id2), leading to transcriptional repression of the *CDH1* gene. Loss of E-cadherin results in the disruption of the polarity complex, a prerequisite for the dissociation and invasion of cancer cells.

Furthermore, our investigations disclose that Foxf2 upregulation is required for TGFβ-mediated apoptosis in NMuMG cells by two major routes: direct transcriptional activation of the pro-apoptotic BH-3 only protein Noxa and negative regulation of EGFR-mediated survival signaling through the repression of its ligands Betacellulin and Amphiregulin. Substantiating the dual function of Foxf2 during EMT found by its implication in cell survival and cell motility, we show that high expression of Foxf2 correlates with good prognosis in early non-invasive stages of tumor development, but with poor prognosis in patients with advanced breast cancer.

In contrast to Foxf2, our studies revealed that the expression of the well established mesenchymal marker N-cadherin is not required to induce EMT in NMuMG cells. Moreover we show that N-cadherin function is not essential to maintain the mesenchymal state and its depletion does not induce the reverse mesenchymal to epithelial transition. In addition, N-cadherin downregulation has no impact on the motility of mesenchymal cells nor on the neurite formation of pancreatic tumor cells. These data indicate that N-cadherin acts as a marker protein without evident function during the EMT process.

In addition to our studies on Foxf2 and N-cadherin, we show that EMT leads to an increase in resistance to anoikis and the capacity to form mammospheres which is thought to correlate with cancer stemness and tumorigenicity. However, we could not detect a defined cancer stem cell subpopulation using cell surface markers nor a general increase of marker expression in mesenchymal cells. These results question whether the correlation of EMT and cancer stemness is intrinsic to certain systems or whether the commonly used surface markers are inadequate to generally detect cancer stem cells.

In summary we identified the transcription factor Foxf2 as important regulator of EMT displaying a dual function in promoting apoptosis as well as invasive properties, while we were not able to detect cancer stemness or a specific function of N-cadherin during EMT. Thus, gaining more and better insights into the molecular function of Foxf2 may provide new strategies for anti-cancer treatments.

Table of contents

1 Introduction

1.1 Hallmarks of cancer

The term cancer describes in a simplified manner a complicated disease in which cells divide without control, leading to the destruction of healthy tissue and spread into other organs by gaining invasive properties. There are different factors, which are able to induce cancer and range from extrinsic factors like UV light, infections or tobacco smoke to intrinsic factors like reactive oxygen species. They can cause DNA damage, leading to alterations in gene expression resulting in silencing of tumor-suppressors and/or the activation of oncogenes. Even though there are various causative factors, resulting in more than hundred different types of cancer, eleven years ago Hanahan and Weinberg were able to pinpoint six hallmarks of cancer that are essential for a cell to acquire on its way to form a malignant tumor (1, 2):

Sustained proliferative signaling

Normal tissue homeostasis includes a careful control of the production and release of growth promoting factors to assure cell growth and proliferation. Cancer cells can overcome this regulation by the use of different mechanisms. They may produce growth factors in an autocrine manner or send signals to activate tumor-associated stroma cells to release growth factors (3, 4). Alternatively, tumor cells elevate the levels of growth factor receptors on the cell surface to become hyperresponsive to the limiting amounts of growth factor ligands (5).

Evading growth suppressors

Tumor cells not only need to induce growth stimulation but also need to circumvent the negative regulation of cell proliferation. This is achieved by inactivating cell-cycle inhibiting pathways blocking *bona fide* tumor suppressors like retinoblastoma (Rb) and p53 or by overexpressing mitogenic proteins such as c-Myc (6). Within normal tissue, cell proliferation is also controlled by contact inhibition to ensure tissue homeostasis. Cancer

cells were found to evade this growth control via contact inhibition by downregulating the expression of Merlin or LKB1 (7, 8).

Resisting cell death

Physiological stresses actuated by extrinsic factors (e.g. Fas ligand/Fas receptor, hypoxia) or intrinsic factors (e.g. genetic instability or signaling imbalances) induce apoptosis in cancer cells. This could be achieved by overexpressing members of the anti-apoptotic Bcl-2 family or silencing tumor suppressors like p53 (9-11). Another important cell-physiological response, usually triggered by nutrient deficiency, is autophagy (12, 13). As a result of uncontrolled and fast proliferation many cancer cells are forced to survive in a nutrient-limited environment. Interestingly autophagy is also a strategy for tumor cells to circumvent metabolic stress by regulation of signaling pathways such as PI3K and/or silencing members of the BH3-only subfamily (e.g. Beclin-1) to restrict necrosis (14, 15).

Inducing angiogenesis

Like untransformed cells, cancer cells require oxygen and nutrients, the supply of which is a limiting factor in fast growing tumors. During progression tumors change their nutrient needs and adjust the steady state from an anti-angiogenic state towards an excess in pro-angiogenic factors, activating the sprouting and growth of the normally quiescent vasculature (16).

Enabling replicative immortality

Untransformed cells are mostly limited in their cell growth and the number of division cycles, leading either to an irreversible non-proliferative but viable state of senescence or to cell death. This limitation is due to the shortening of the telomeric DNA (17). The expression of the telomere extending enzyme telomerase leads to spontaneous immortalization of tumor cells (18).

Activating invasion and metastasis

Solid benign tumors form tight junctions with neighboring cells and adjacent tissues. By breaking down these adherens contacts, tumor cells gain invasive capabilities and

progress to higher pathological grades of malignancies resulting in metastasis formation in distant organs (19). For more details see Chapter 1.3 Cancer cell migration.

1.2 Cancer gene signatures

Work on organ-specific metastasis provides an indication for the existence of metastasis promoting genes. Therefore cancer-associated gene expression profiling serves as a good tool to classify tumor subtypes, discriminate disease etiology, predict the relapse risk, identify genes that mediate disease progression and select ideal therapeutic options to avoid resistance. Among solid tumor types, breast cancer has been a prominent target in this kind of analyses (20).

1.2.1 Breast cancer signatures

Breast cancer is the top cancer in women both in the developed and the developing world. This cancer shows heterogeneous characteristics with a high variability in patient survival rates, depending on the disease subtype. The availability of clinical samples from large patient cohorts and the existence of different therapeutic options made breast cancer a convenient subject for prognostic gene expression signature research (20). Breast cancers can be classified according to several signatures, such as the intrinsic subtype signature, obtained by unsupervised clustering of breast tumors depending on their gene expression profile (21) By this they are classified in the most frequent luminal subtype, which is divided into two subclasses (A and B) that express oestrogen receptor (ER) and/or progesteron receptor (PgR). Subclass A is usually HER2 negative while subclass B is more likely to be HER2 positive. The more aggressive basal subtype is hormone receptor negative and in many cases Brca1-deficient and/or HER2-negative (triple-negative tumors) (22, 23). The fast proliferating triple-negative tumors are lacking the receptors and are therefore resistant to the existing ER, PgR and HER2-targeted therapies (24). Although widely used, the term basal-like breast cancer led to some confusion due to the unclear definition of their cytokeratin expression status to discriminate these tumors from luminal cancers and is therefore debated (25). The fourth subtype overexpresses the human epidermal growth factor-2 (HER2 or ErbB2) and these tumors are prone to early and frequent relapse and to distant metastasis (26). The normal breast-like subtype are the tumors that do not fall into any of the other categories and usually have a good prognosis (27). Each subtype is characterized by a distinct

histopatology and survival rate (28). Other signatures were obtained from groups of tumors with known clinical outcome, like for example the 76-gene poor-prognosis signature (29) or signatures derived from functions that are associated with tumor progression, like the hypoxia signature (30). A more recently identified molecular subtype of triple-negative breast cancer is the claudin-low subtype, which is characterized by the expression of mesenchymal and stem cell-associated markers and which correlates with poor prognosis (31). Classification of tumors according to these different gene-signatures offers additional predictive values for the therapeutic assessment.

1.3 Cancer cell migration and metastasis formation

Cancer cell metastasis rather than the primary tumor itself is the leading cause of death of cancer patients worldwide. The spread of cancer cells from the primary tumor to a secondary site within the body is a persisting challenge in battling this life-threatening disease. Malignant cells must accomplish different steps to metastasize. These are the dissociation of tumor cells from the primary tumor, invasion in the adjacent tissue, intravastion into lymphatic or blood vessels, dissemination through the vasculature and finally the extravasation and nesting in distant organs (Figure 1) (32). Each of these steps requires particular attributes and molecular programs to adapt to the different environments and conditions. To leave the solid primary tumor cancer cells need to abrogate the tight cell-cell compound and remodel the cell-matrix adhesion to invade the adjacent tissue. These initial steps involve cellular strategies, which can be divided based on histological criteria in two fundamentally different patterns of invasion (i) single migrating cells and (ii) collective cell invasion.

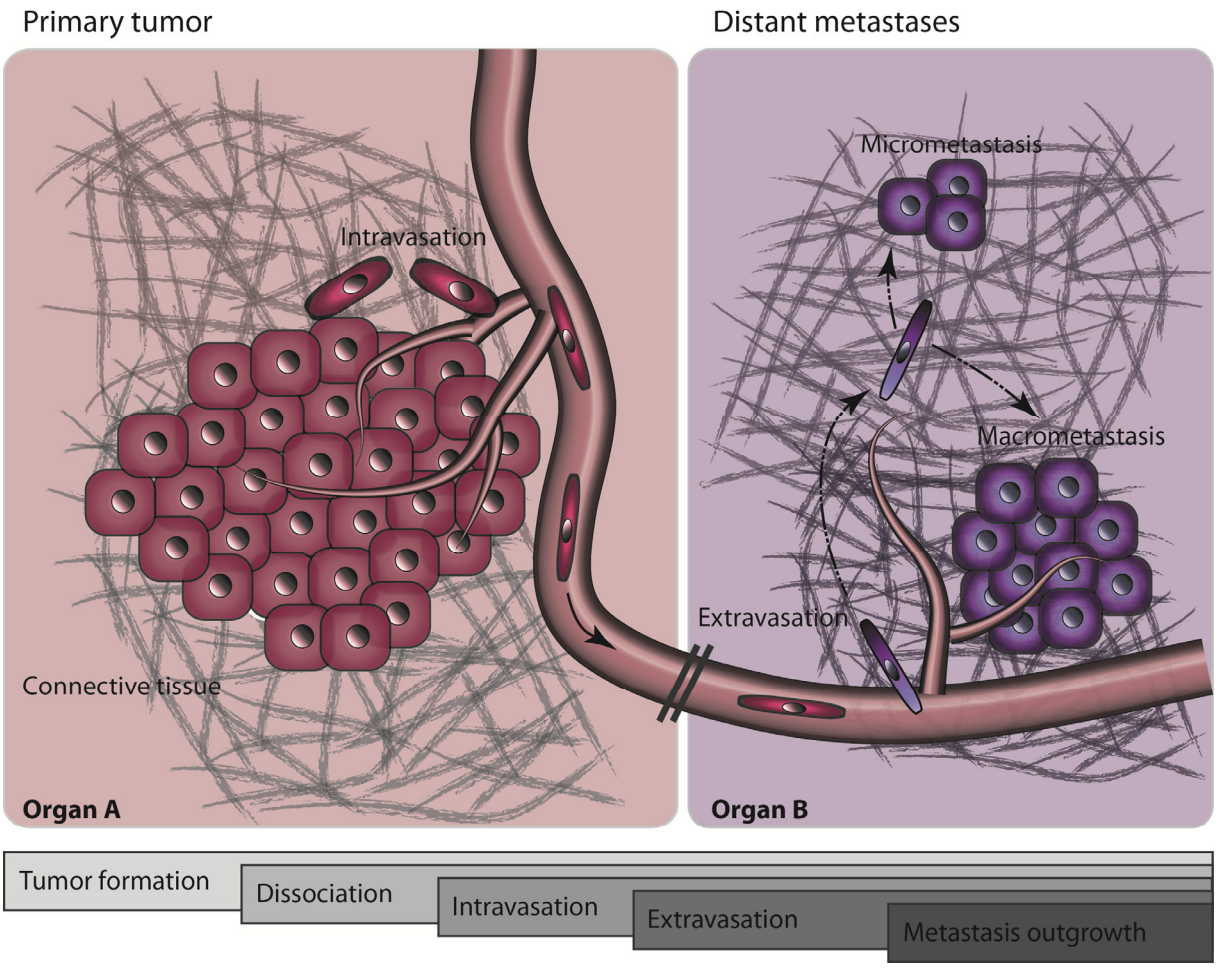


Figure 1: Metastasis formation. Acquisition of unlimited proliferation potential and resisting cell death predates benign tumor formation. Formation of new blood vessels provides the growing tumor with nutrients and oxygen. During progression to the malignant stage, tumor cells acquire migratory and invasive properties and invade the adjacent tissue. Angiogenesis and lymphangiogenesis provide a route for tumor cells to enter the circulation and colonize distant organs.

1.3.1 Single cell invasion

Single cell migration is characterized in histology by the existence of isolated and scattered tumor cells in the surrounding tissue. Cancer cells can follow two distinct strategies to migrate as single cells: a fibroblastoid (mesenchymal) or an amoeboid pattern (Figure 2A,B).

1.3.1.1 Fibroblastoid migration

It has been shown that single cells leaving a tumor at its periphery in many cases migrate with a mechanism that involves the loss of epithelial polarity and the achievement of mesenchymal morphology during Epithelial to Mesenchymal Transition (EMT) (33).

Essential characteristics of EMT are the disruption of the tight cell-cell contacts, the acquisition of a fibroblastoid spindle-shape morphology, the gain of invasiveness and cell-stroma interaction as well as a slow division rate, resulting in the release of single cells from the solid epithelial tumor (34). Mesenchymal cell migration is not only exerted by cancer cells but also by normal untransformed cells, such as fibroblasts, endothelial cells and smooth muscle cells, and is based on cellular movements driven by a leading edge with Rac-induced cell protrusions and formation of a focalized actin cytoskeleton with the formation of so called stress fibers. These spindle-like shaped cells remodel the extracellular matrix structure by proteolytic reorganization and exhibit a slow turnover of integrin-mediated focal adhesions resulting in a rather slow cell migration with velocities ranging from 0.1 to 1 µm/min (35, 36). The EMT phenomenon was first discovered in cell cultures and the actual occurrence of EMT and its role in the progression of epithelial cancers (carcinomas) in patients is still highly debated. In most cancers in fact, full EMT, which means the complete loss of epithelial markers and the acquisition of mesenchymal markers, is rarely seen (37). As these evading cells acquire more mesenchymal traits it becomes more difficult for the pathologist to distinguish these cells from the mesenchymal cells surrounding the tumor. Nevertheless, there is more and more evidence that EMT at least to some extent plays a role in cancer cell metastasis even though most cancer cells were seen to only undergo partial EMT, retaining epithelial cytokeratin expression but gaining mesenchymal markers such as Vimentin (38). In fact, the expression of Vimentin in epithelial cells of breast tumors correlates with a shorter post-operative survival of patients (39). Furthermore, nuclear β-catenin - another characteristics of EMT - is found in colorectal cancer distributed along the invasive front but not in the tumor center (40). The highly invasive metaplastic breast cancer (MBC) and the claudin-low tumors, have both been observed to display EMT attributes such as an attenuated E-cadherin expression as well as elevated levels of the EMT inducing transcription factors Twist and Slug (31). These data suggest that EMT is an important process to dissect in order to decode the mechanistics behind cancer cell migration.

1.3.1.2 Amoeboid migration

Some cell types, such as circulating stem cells, leukocytes and tumor cells of haematopoietic origin show a more diffuse structure of the actin cytoskeleton and use more

short-lived and less adhesive cell-matrix interactions (41). This fast-gliding manner of migration is independent of integrins and proteolytic activity, focal contact formation and stress fibers. The movement is mainly driven by RhoA/ROCK-mediated bleb-like protrusions with active myosin-actin contractions, and the cells squeeze through gaps in the ECM. The low adhesion attachment to the substrate allows the cells to translocate much faster in the 3D environment (35). Amoeboid and mesenchymal migration are two modes that are mutually interchangeable. Suppression or increase of the activity of the molecular pathways underlying either of the modes can cause a switch to the other type of migration. This change is called amoeboid-mesenchymal transition (AMT) or mesenchymal-amoeboid transition (MAT). MAT/AMT is triggered by rapid alterations in the migratory mode that arise as a reply to the current prerequisites of the environment. The transition may play a role in different stages of the metastatic process, when a specific environment requires adaption of the tumor cells (42).

1.3.2 Collective cell migration

In contrast to individual cell migration, collectively migrating cells retain their cell-cell junctions by the continuous expression of adhesion molecules and migrate in sheets, strands, tubes or clusters, either remaining connected to the primary tumor (coordinated invasion) or as detached cell groups or clusters (cohort migration) (Figure 2 C/D). This type of migration is mainly observed in basal cell, oral squamous cell and some other epithelial carcinomas (43-45). Similar to single invasive cells, collectively migrating cells form membrane protrusions like ruffles and pseudopodes and form integrin-mediated focal adhesions connected to the actin cytoskeleton. They apply pericellular proteolysis to generate a path through the extracellular matrix scaffold and they use actin-myosin contraction for cell movement (46). Collective migrating cells do not retract their cellular tails but rather exert mechanical forces like pulling on adjacent cells that are connected by adhesion junctions (35). The clustered cohort-like cancer cell dissemination seems to be highly efficient in embolizing lymphatic or blood vessels and in surviving in the circulation (47). This coordinated type of invasion is typically seen in squamous cell carcinomas, melanoma and breast and colon carcinomas. To penetrate the extracellular matrix, the leading cells generate an invasion path by the use of β_1-integrin-mediated focal adhesions

and local expression of MMP14 at their leading edges to cleave the collagen fibers and orient them in tube like structures in which the following cell mass is able to migrate (48, 49). Studies in our laboratory have also detected the expression of Podoplanin, a transmembrane glycoprotein, implicated in the remodeling of the actin cytoskeleton, on the cell surface of collectively migrating cells (50). Podoplanin expression is mainly found in the outer layer of the invading front and forced expression of Podoplanin in epithelial breast cancer cells and in human keratinocytes induces cell migration and invasion with filopodia formation without the downregulation of E-cadherin and a significant decrease of cellular stress fibers. These findings indicated that Podoplanin is able to shift the invasion pattern from an EMT driven single-cell invasion to collective invasion in the absence of EMT.

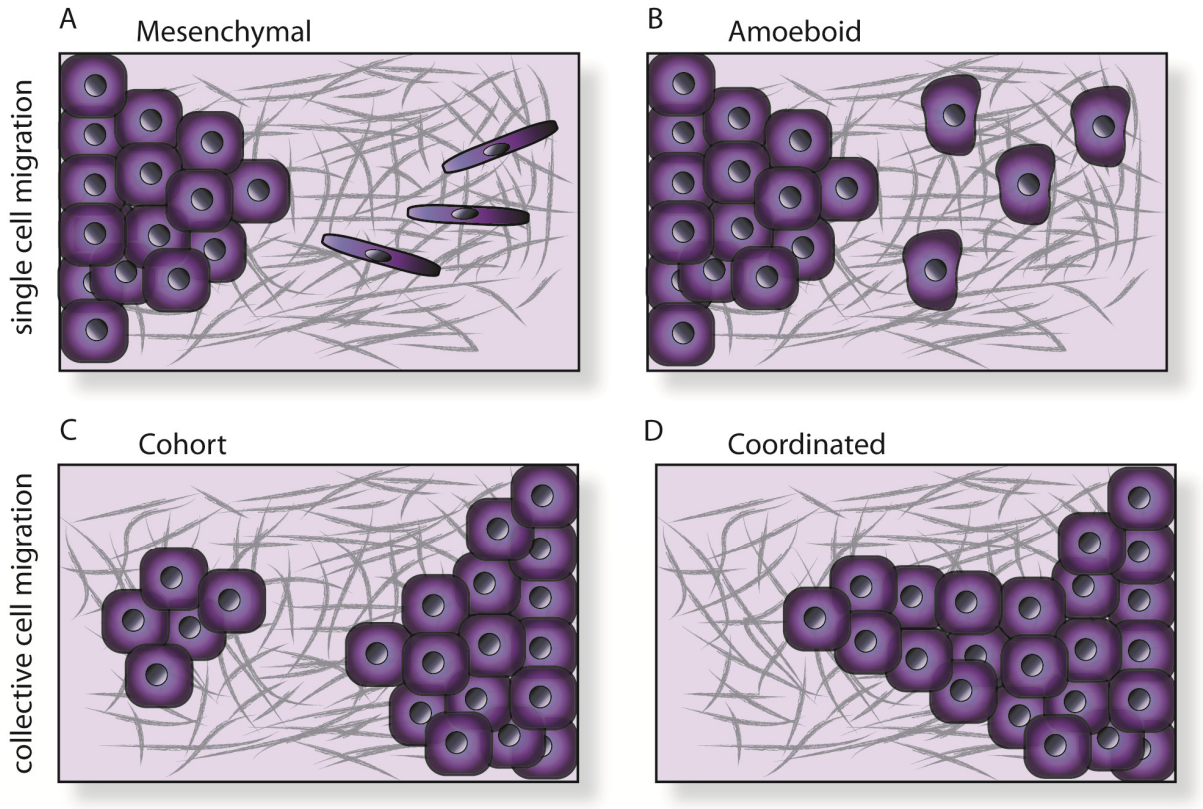


Figure 2: Different strategies of tumor cell invasion. (A) Individual cancer cells detach at the invading front from the primary tumor by undergoing an epithelial to mesenchymal transition (EMT) to break down the tight cell-cell contacts and gain a spindle-like morphology. The extracellular matrix (ECM) structure is remodeled by proteolytic reorganization. (B) The amoeboid invasion is characterized by weak interactions with the ECM and the independency of proteases and calpains. The invading cancer cells are able to switch between the amoeboid and the mesenchymal invasion (MAT/AMT), depending on the prerequisites of the microenvironment they meet. (C) The cohort migration is characterized by the collective detachment of a migrating cell nest from the primary tumor invading the ECM by proteolytic degradation. (D) In the coordinated collective cell invasion type cancer cells invade the surrounding tissue by remaining their tight cell-cell contacts to the neighbor cells as well as to the primary tumor.

1.3.3 Tumor dissemination through the vasculature and extravasation

Although the rate of disseminating tumor cells increases with primary tumor size, shedding of malignant cells can already occur in the early onset of the tumor (20).Tumor cells exhibit a high plasticity in adapting their type of invasion in order to progress through the various stages of the metastatic process. Collectively migrating cells need to break away from their neighbors to be able to enter the bloodstream. Therefore, tumor cells have to undergo adaptive switches by activating different programs like EMT, depending on the different environments. Migratory cancer cells that undergo EMT acquire specific properties like the expression of VE-cadherin, which allow them to interact with endothelial cells and enhance the capacity of transendothelial migration (51, 52). Furthermore, the widely observed increase in microvessel density as well as the leakiness of the tumor vasculature facilitates the entry of cancer cells into the circulation. Surviving in the bloodstream necessitates acquisition of qualities, such as the evasion of detachment-triggered cell death (anoikis) as well as surviving the shear forces and evade attacks from the immune system (53). To be protected from immunological assault, some tumor cells induce platelet aggregation, which has also been reported to facilitate tumor cell cluster arrest in the microvasculature (54). One of the factors identified to induce platelet aggregation in some human cancers, is the transmembrane protein Podoplanin (55), which was already identified to play an important role in collective cell migration.

1.3.4 Extravasation of circulating tumor cells and establishment at secondary sites

Gains of invasive characteristics as well as angiogenesis are important events for metastasis albeit they are not sufficient themselves. Outgrowth of metastases in distant organs depends on whether some circulating tumor cells - generally only a small minority of them - manage to enter the parenchyma of a target organ (20). To do so, cancer cells need to extravasate from the blood stream by first adhering to the vascular endothelium and then transmigrate through the endothelial cells to invade the surrounding tissue. The mechanisms underlying this extravasation process is still poorly understood. Nevertheless, recent studies have identified a lung metastasis gene signature in breast cancer, whereof the combinatorial expression of four genes, namely the EGFR ligand Epiregulin, the cyclooxygenase COX2 and the matrix metalloproteinases MMP1 and 2, were found to not only facilitate the formation

of new tumor blood vessels and the entry into the circulation but also to advance the exit from the bloodstream at the lung capillaries (56, 57). Furthermore VEGF, an angiogenic factor, which is expressed by most cancer cells, increases the permeability of the endothelium by uncoupling endothelial cell-cell junctions and thereby potentiating tumor cell extravasation (58). The dissemination of tumor cells has characteristic patterns of organ tropism depending on the cancer type. The precise mechanism of this homing process still needs to be established, although a number of chemokines have been implicated to play a crucial role in organ-specific metastasis. Breast cancer is characterized by a distinct metastatic pattern, involving regional lymph nodes, bone marrow, lung and liver. It has been shown that in malignant breast cancer, the chemokine-receptors CXCR4 and CCR7 are expressed at high levels in the primary tumor as well as in the metastases. Their respective ligands SDF1α and CCL21 are abundant in organs representing the first target location for breast cancer metastasis (59). This finding implicates that specific expression of a set of genes may guide circulating tumor cells to distinct secondary sites.

1.3.5 Preparing the pre-metastatic niche

Another interesting hypothesis to explain the tissue-specific pattern of metastatic progression in cancer, is the formation of a permissive microenvironment for incoming tumor cells, by bone marrow-derived cells (pre-metastatic niche) (60). Labeled bone marrow-derived cells have been observed to specifically home and form clusters at common sites of metastasis before the arrival of tumor cells. These bone marrow-derived cells (BMDCs) express VEGFR-1 and other hematopoietic markers to maintain their progenitor status and preferentially localize at Fibronectin-rich areas. Interestingly, the preparation of the pre-metastatic niche is regulated by the primary tumor. Expression of high levels of VEGF mobilizes and recruits VEGFR-1-expressing hematopoietic progenitor cells (HPC) resulting in increased HPC clustering and formation of metastasis at multiple sites (61). Moreover the secretion of tumor specific growth factors like VEGF-A, TNFα and TGFβ induces the expression of inflammatory chemoattractants, which in turn attract myeloid cells at distant pre-metastatic sites (62). By this a tumor-specific chemokine profile can prime and direct the location of future metastatic tumor growth. More recently, the ECM crosslinking enzyme LOX was identified as an important factor in the formation of the pre-metastatic niche. The

secretion of the enzyme into the circulation by the hypoxic environment of primary breast tumors leads to a co-localization of LOX with Fibronectin at future metastatic sites and crosslinking of ECM components like collagen IV. Thereby attracted myeloid cells initiate a positive feedback loop by further cleavage of collagen IV and a recruitment of BMDCs and metastasizing tumor cells (63). These findings indicate that the initial deposition of Fibronectin and LOX in the pre-metastatic niche contribute to the formation of an appropriate ECM that facilitates the recruitment of BMD and other mesenchymal cells to prepare the suitable environment for future metastasis (64). Targeting the factors that initiate the pre-metastatic niche could be a novel approach to effectively treat cancer cell metastases and therefore improve patient survival.

1.4 EMT

The expression Epithelial to Mesenchymal Transition (EMT) describes a complex molecular and cellular program by which epithelial cells abandon their differentiated features, such as cell-cell adhesion, planar and apical-basal polarity and low cell motility and acquire mesenchymal characteristics, including motility, invasiveness and increased resistance to apoptosis (Figure 3) (65). EMT is implicated in several physiological as well as pathological processes, as a critical mechanism for embryonic development and is re-engaged in adults during wound healing, tissue regeneration, organ fibrosis and cancer progression and metastasis (66). The biological need for EMT is to produce cells with distinct morphology and functions to acquire motile and locally invasive capabilities and to enable genetically unstable tumor cells to disseminate. EMT can be classified into three types, depending on the biological context in which they proceed (67).

Type 1 EMT is the subtype associated with implantation, embryogenesis and organ development and is the only subtype, which is independent of inflammatory processes. Type 1 EMT does not result in systemic spread of mesenchymal cells via the circulation but rather generates mesenchymal cells, which have the potential to subsequently undergo the reverse process (MET) to redifferentiate and form secondary epithelia.

The second type of EMT is a repair-associated incident provoking wound healing, tissue regeneration and organ fibrosis. This type 2 is clearly inflammation dependent and

ends when the inflammatory process diminishes. Ongoing inflammation causes persisting EMT, which can result in organ destruction.

The EMT associated with cancer progression and metastasis is classified as type 3 EMT. This subtype occurs in neoplastic cells and may go on to variable extents, resulting in groups of cells retaining epithelial characteristics while gaining some mesenchymal ones and other cells undergoing full EMT. The specific signals inducing EMT in cancer cells still need to be elucidated but the genetic and epigenetic alterations occurring during cancer progression result in a high responsiveness to EMT inducing signals, which are contributed by the tumor stroma.

The actual occurrence of EMT in the process of metastasis in patients is still highly debated, however recent studies implicate that primary tumors displaying an EMT-like gene expression profile are more likely to be associated with distant metastases formation and worse prognosis for overall survival (68-70). In contradiction to these findings are the frequent observations that distant metastases from carcinomas are formed by cells displaying an epithelial phenotype highly similar to the cancer cells of the primary tumor (68, 69). This discrepancy can be explained by the highly dynamic nature of the EMT program. The disseminated mesenchymal cancer cells undergo the reverse process (MET) after metastatic spread and colonization, to revert to a more differentiated, epithelial cell state enabling them to establish in the distant location (71). There is increasing evidence that EMT and its reverse process MET are pivotal regulators of cell plasticity in carcinomas and play important roles in drug resistance, relapse and metastatic progression (72).

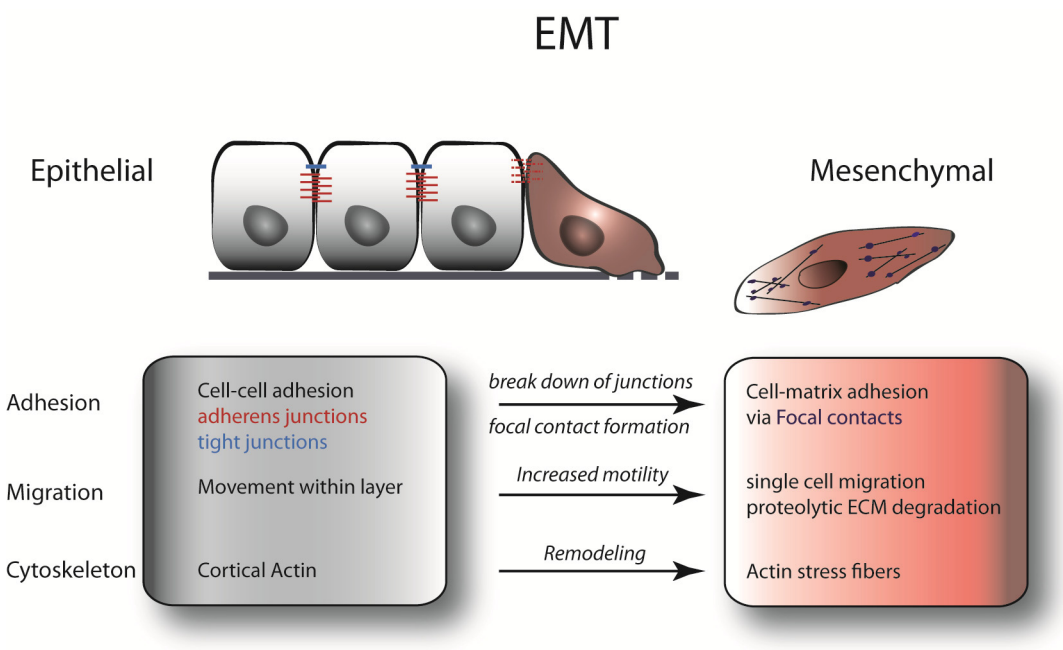


Figure 3: The transition from the epithelial to the mesenchymal state is accompanied by specific morphological changes. EMT occurs when epithelial cells break down their cell-cell junctions and lose their apical-basal polarity and acquire mesenchymal characteristics such as focal adhesions, actin skeletal reorganization to stress fibers and increased invasiveness.

1.4.1 Epithelial cell-cell adhesion

Epithelial cells naturally form stable sheets containing of apical-basal polarized cells with tight cell-cell contacts formed by tight junctions (TJ) and adherens junctions (AJ) and desmosomes (73). Tight junctions are located at the apical side, forming an intercellular sealing to avoid paracellular diffusion. Among the main components building the TJ are the transmembrane proteins Occludin and Claudins as well as the cytoplasmic molecules Zonula Occludens (ZO) 1,2,3 and p120. The cytosolic part of ZO proteins contribute to the strength and integrity of the TJ by attaching to the actin filaments (74). Located adjacent to the tight junctions are the adherens junctions, forming a belt-like structure at the lateral interface of epithelial cells. The single-span transmembrane glycoprotein E-cadherin is the prototype member of the adherens proteins. E-cadherin interacts with neighboring cells by forming calcium-dependent homodimers with their cadherin repeats. The cytoplasmatic domains of E-cadherin are anchored to the cytoskeleton by binding directly to β-catenin, which is linked to the actin filaments via α-catenin (75). Thus, the formation of cadherin mediated cell-cell interaction not only functions to establish tight cell-cell adhesion but also to define the

adhesive specificity of the cell and to modulate the organization of its cytoskeleton. The juxtamembrane domain of E-cadherin is bound to another catenin, namely p120, which assures surface tracking, the proper membrane localization of E-cadherin and its lysosomal degradation (76-78). Moreover, p120 is involved in the stability of epithelial cell-cell adhesion via the repression of RhoA GTPase activity and activation of the GTPases Rac and Cdc42. These key-regulators of actin assembly play a major role in the stability of cell-cell adhesion by fortifying actin stress fibers (RhoA) and activate the formation of membrane protrusions like lamellipodia and filopodia (Rac and Cdc42) (79, 80). In addition to its adhesive function, E-cadherin comprises also signaling capacities, transduced by the intracellular domain or its interacting proteins, such as β-catenin or it forms multimeric complexes with receptors such as c-Met, IGF1R or integrins (81). Notably, proteolytic cleavage of the intracellular domain of E-cadherin by γ-secretase produces a C-terminal cytoplasmic fragment (Ecad/CTF2). This Ecad/CTF2 is imported into the nucleus, where it modulates the interaction between the suppressive complex of p120-catenin and the transcriptional repressor Kaiso, which is involved in the regulation of the Wnt signaling pathway (82). The nuclear localization of Ecad/CTF2 is specifically enhanced by p120-catenin, where it forms a trimeric complex with p120 and Kaiso, implicating a role of Ecad/CTF2 in the regulation of gene transcription (83). These findings expand the field of activity of E-cadherin beyond its adhesive functions and punctuate the critical capacity of E-cadherin as a regulator of signaling complexes.

1.4.2 Mechanisms of EMT activation

The process of EMT can be triggered by diverse intrinsic signals, such as gene mutations, as well as extrinsic stimuli including growth factor signaling, extracellular stroma interaction and hypoxia. The downstream intracellular signaling of EMT includes transcriptional reprogramming and various positive feed-back loops (84). Among the EMT-inducing growth factors, the members of the transforming growth factor β (TGFβ) family are the best characterized inducers. They are involved in EMT during embryonic development, wound healing and cancer progression (65, 85). Receptor-mediated signaling can also be activated by various other factors, such as the hepatocyte growth factor (HGF) (86), members of the epidermal growth factor (EGF) (87) and of the fibroblast growth factor (FGF)

family. In detail, hepatocyte growth factor (HGF) and its cognate receptor c-Met are well known inducers of cell scattering, migration, invasion and EMT and were found to be mutated in a variety of human cancers (88). The cooperation of c-Met with the v6-splice variant of the hyaluronan receptor CD44, which is frequently upregulated in cancer and was implicated in tumor metastasis, seems to be required for HGF-induced c-Met stimulation with subsequent MEK and MAPK activation (89, 90). All the above mentioned inducers of EMT activate the disassembly of junctional complexes and changes in cytoskeletal organization as well as the activation of transcriptional regulators accompanying this process. A central target of these changes in gene-expression patterns is E-cadherin, which has a direct relevance in the onset and continuation of EMT. E-cadherin expression is also targeted by genetic or epigenetic changes occurring during or activating EMT, resulting in heritable effects and a stable mesenchymal phenotype. An important activator of EMT is hypoxia, which is often associated with fast growing tumors. Low O_2 levels activate the various hypoxic programs including hypoxia-induced factor-1α (HIF1α), HGF, Notch or NFκB pathway activation, resulting in the induction of the EMT-inducing transcription factor SNAI1 (91, 92). With increasing interest in non-coding RNAs, miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) and miR-205 have been identified as regulators of EMT and metastasis. The regulation of EMT by non-coding RNAs has been found to be coupled to the repression of E-cadherin expression by targeting the transcription factors ZEB1 (δEF1) and ZEB2 (Sip1) (93, 94). Another recently found potential EMT-inducing mechanism is alternative splicing, which can generate isoforms of the same gene with antagonistic functions. Two RNA binding proteins, the epithelial splicing regulatory proteins 1 and 2 (ESRP1 and ESRP2) were recently identified to control the splicing of epithelial-specific isoforms of EMT-associated genes, such as FGFR2, CD44 and p120-catenin (95, 96). This complexity of factors and pathways involved in the induction and continuation of EMT explains why EMT is not a simple change in cell shape or adhesive capabilities but rather a modification of all fundamental cell programs in a highly dynamic manner.

1.4.3 Loss of E-cadherin function

An early event during EMT is the disassembly of tight junctions resulting in the disruption of the polarity complex and initiation of cytoskeleton reorganization (97, 98). Loss

of adhesive function of E-cadherin is frequently observed in clinical and experimental studies, occurring in most human epithelial cancers and is therefore a clinical indicator for poor prognosis (19, 99). Given that E-cadherin is an important regulator of epithelial cell shape and tissue homeostasis, its expression is accurately controlled and its loss can be caused by a variety of genetic as well as post-translational modifications during tumor progression.

1.4.3.1 Genetic and epigenetic control of E-cadherin

Stable alterations of gene expression occurring during cancer progression include gene mutations and deletions, copy-number aberrations or chromosomal rearrangement as well as epigenetic changes, like covalent modification of the DNA (72). Although the suppression of CDH1, the gene encoding E-cadherin, is usually achieved by post-translational or epigenetic modifications, inactivating mutations have been identified in hereditary gastric cancer (100). Furthermore, lobular breast carcinomas frequently show somatic genetic inactivation of the CDH1 gene with an indication that this is as an early event during sporadic tumor development (101). Another important repressing mechanism of E-cadherin is the hypermethylation of its promoter CpG islands, which can act as a second hit for transcriptional repression (102). This modification is associated with the recruiting of methyl DNA binding proteins (MBDs) as well as histone deacetylase activity (HDACs), which in conjunction result in the compaction of the promoter region and, thus, to the inactivation of the gene (103). Epigenetic silencing of tumor suppressor genes is also achieved by histone modifications such as acetylation, phosphorylation, ubiquitination, sumoylation and methylation, each of these with specific consequences for chromatin function, of which lysine methylation has been shown to play a crucial role in the control of gene expression (104, 105). The well described E-cadherin repressor Snail1 has been found to associate with the polycomb repressor complex 2 (PRC2) members Suz12 and Ezh2 and to recruit this complex to the CDH1 promoter region, thus promoting the repression of E-cadherin by trimethylation of lysine 27 in histone 3 (H3K27 me^3). These epigenetic changes appear to be dynamic during tumor progression, as hypermethylation of the CDH1 gene is frequently found in invasive and metastatic tumors. Yet, it has been shown that re-expression of E-cadherin at secondary sites promotes survival of the cancer cells (106, 107).

1.4.3.2 Transcriptional control of E-cadherin silencing

Besides the regulation of the CDH1 promoter via hypermethylation, direct transcriptional control by a list of transcription factors, such as Snail1, Slug (Snail2), ZEB1 (δEF1), ZEB2 (Sip1), E47 and Twist have emerged as important regulatory mechanism of E-cadherin expression (108). The expression of these repressors is controlled by diverse signaling such as the activation of the TGFβ, HGF, EGF and Notch pathways and furthermore, they show mutual regulation via positive and negative feedback loops. The above mentioned transcriptional repressor Snail1 was also found to bind directly to the E2-boxes in the E-cadherin promoter to regulate its expression (109). Furthermore, Snail1 induces the expression of the zinc finger transcription factor ZEB1, which interacts with the CtBP co-repressor to suppress E-cadherin (110, 111). ZEB2, another member of the homeodomain and zinc finger family, was also found to be coexpressed with Snail1. In contrast to Snail proteins, ZEB1 and ZEB2 are able to interact with transcriptional co-activators such as p300 and pCAF, indicating a fundamental different mechanism of Snail and ZEB transcription factors to activate mesenchymal markers (112). A more indirect mechanism of transcriptional regulation is observed for the members of the inhibitor of differentiation (Id) protein family. These proteins were found to function as negative regulators of E-protein or basic helix-loop-helix (bHLH) transcription factors by the formation of active heterodimers, since they are lacking an own DNA binding domain (113). These data indicate that different repressors participate in E-cadherin silencing using diverse mechanism depending on cancer type or the stage of tumor progression (103).

1.4.3.3 Post-translational control of E-cadherin

A more dynamic way of E-cadherin control happens on the post-translational level such as O-glycosylation of the freshly synthesized E-cadherin to prevent protein transport to the membrane (114) as well as the degradation of mature membrane-bound E-cadherin by proteolytic cleavage or endocytosis (115, 116). As described in chapter 1.4.1, proteolytic cleavage of E-cadherin by γ-secretase can result in the release of the active fragment Ecad/CTF2, which is subsequently transported to the nucleus to interfere with the transcriptional repressor complex p120 and Kaiso, affecting survival (83). Furthermore, the E-cadherin adhesion complex is a target of receptor tyrosine kinase or Src-mediated

phosphorylation, leading to ubiquitylation by the H3 ligase Hakai followed by degradation (117). The downregulation of E-cadherin not only entails the disassembly of the adherens junctions but also releases cytoplasmic members of the adhesion complex, such as β-catenin and p120-catenin, to administrate ambivalent functions controlled by their subcellular localization (32). This complexity and diversity of E-cadherin regulation indicates that the cells need to have dynamic range of E-cadherin expression, depending on the environmental and competence requirements.

1.4.4 The cadherin switch

E-cadherin, the prototype family member of epithelial Cadherins, is important for epithelial structure maintenance, whereas the mesenchymal N-cadherin is expressed in nervous tissue, connective tissue and migratory cells. The cadherin switch is a hallmark of EMT resulting in alterations of adhesive properties, acquiring affinity for mesenchymal cells, such as fibroblasts or vascular endothelial cells and enhancement of migratory and invasive capabilities (118, 119). Change from E-cadherin to N-cadherin expression occurs during development in mesoderm formation, but it is also suspected to support the transition from benign to invasive malignant tumors (120). In most, if not all, epithelial cell-derived tumors, loss of E-cadherin-mediated cell-cell adhesion is associated with increasing malignancy, achieving the prerequisites for dissociation and invasion of cancer cells (121). Indeed in some human tumors, a *de novo* expression of N-cadherin or Cadherin-11 is observed subsequent to E-cadherin silencing and correlates with poor prognosis (119, 122-124). Although N-cadherin has been shown to promote motility and migration, the functional implication of this switch could be more diverse than first expected in tumor progression and play a role in the process of new homing and altered affinity to neighboring cells (98, 125, 126). The regulation of N-cadherin expression still needs to be elucidated in detail, yet recent studies showed an activation of N-cadherin via collagen I as well as transcriptional upregulation of the mesenchymal protein by the transcription factor Twist, in a β_1-integrin-dependent manner (127, 128). Like its epithelial counterpart E-cadherin, N-cadherin is coupled to the cytoskeleton via α-catenin and β-catenin, displaying cell adhesion properties by the formation of transcellular homodimers as well as signaling functions by interacting with signal transduction molecules, such as the PDGFR, which is known to induce actin

reorganization, proliferation and differentiation, playing an important role in EMT (129-131). Furthermore, our laboratory has shown a physical association of N-cadherin with the fibroblast growth factor receptor (FGFR) forming a complex together with the cell adhesion molecule NCAM (132, 133). This functional cooperation stabilizes the receptor by preventing its internalization and therefore results in sustained MAPK activity, increased cell motility and MMP secretion, promoting invasiveness (134, 135). Additionally, proteolytic processing of N-cadherin by ADAM10 and γ-secretase initiates the shedding of extracellular domain and release of the cytoplasmic fragment (Ncad/CTF2) acting as a transcriptional repressor. Binding of the Ncad/CTF2 to CBP leads to the induction of its proteosomal degradation and by this interferes with the CBP/CREB transcription complex and the expression of genes, which are important for proliferation and differentiation, such as c-Fos (136). Interestingly, N-cad/CTF2 conveys also pro invasive β-catenin/Tcf signaling by diverse contributions, as there is the inhibition of β-catenin phosphorylation, upregulation of β-catenin transcription and reduced sequestration of β-catenin at the cell-cell junctions as a result of reduced protein levels of full-length N-cadherin (137). Faced by this pleiotropic functions, N-cadherin plays a critical role in the regulation of EMT and invasiveness. Besides executing cell adhesion functions and determination of mesenchymal cell affinity, it is involved in distinct signaling pathways, altering the migratory capability of the cell by modulation of growth factor signaling and actin cytoskeleton remodeling.

1.4.5 The role of integrins in invasion and EMT

The extracellular matrix (ECM) is a highly dynamic structure, formed by a variety of proteins and proteoglycans, involved in cellular processes such as proliferation, survival, differentiation and migration. The control of these processes is mainly mediated by interactions of the cellular adhesive adaptors, integrins and the ECM. Integrins are heterodimeric transmembrane receptors, formed by α- and β-subunits, executing similar to the cadherins mechanical adhesion as well as signaling functions. The inactive, low ligand affinity confirmation can be activated by binding of intracellular proteins like talin or by MMP-mediated proteolytic cleavage (138, 139). Integrins are linked to the actin cytoskeleton via Parvin or a Paxillin/Vinculin/Parvin complex, triggering the activation of diverse signaling cascades and their effector proteins, such as MAPK, NFκB, Jun, β-catenin and others, via

direct targets like FAK, Src-family kinases and PKB (140, 141). During EMT integrins display distinct functions including the initiation and enforcement of EMT and invasion. In pancreatic cancer integrin $\alpha_1\beta_1$ as well as $\alpha_2\beta_1$ were found to induce loss of E-cadherin-mediated cell-cell contacts and activation of the β-catenin/TCF pathway by binding to Collagen I (142). Furthermore, as described in chapter 1.4.4, activation of $\alpha_2\beta_1$-integrin by Collagen I together with the collagen receptor discoidin domain receptor (DDR1) leads to the upregulation of N-cadherin expression (127). This induction of the cadherin switch plays an important role in the initiation and progression of EMT. Moreover, Snail, a well known inducer of EMT, is able to activate the expression of the pro-invasive $\alpha_v\beta_3$-integrin, which is mainly located at the invading cancer front (143, 144). Other studies showed that the co-localization and cooperation of β_1-integrin and membrane-type matrix-metalloproteinase 1 (MT1-MMP) is essential for tumor cell invasion into a collagen matrix and that both proteins are implicated to have important roles in EMT (145-147). Furthermore, the interaction of MT1-MMP and $\alpha_v\beta_8$-integrin leads to the activation of TGFβ, one of the most potent inducers of EMT (84), indicating diverse contributions of the integrin family to this complex process .

1.4.6 EMT in cancer metastasis

The EMT transdifferentiation program was first delineated as a cell culture phenomenon and the relevance of EMT in physiological processes and cancer progression has remained a matter of debate until very recently (72). The long believed theory of metastasis as a late event during tumor progression is refuted by the finding of a systemic spread of tumor cells from early lesion in HER-2 transgenic mice as well as in human ductal carcinomas (148). In clinical samples, EMT may be a focal event that is easily overseen as it is highly dynamic. Moreover the individual tumor-derived mesenchymal cells invading the surrounding tissue are delicate to distinguish from stromal cells or tumor associated fibroblast (149). Nevertheless, there is morphological evidence of EMT at the invasive tumor fronts of many human tumors, like in colon carcinoma, where single migratory cells show an EMT phenotype, with loss of E-cadherin, deregulation of the Wnt-pathway and a selective loss of the basement membrane (150). This phenomenon is also found in other solid tumors like breast carcinomas, where EMT is associated with histological grades, the basal-like

phenotype and the metaplastic subtype, correlating with a decrease in relapse-free survival (149, 151-154). Although EMT is observed at the invading front of epithelial cell-derived cancers, there is only few data demonstrating the presence of EMT in circulating tumor cells (CTCs). Recent studies in human non small cell lung cancers (NSCLC) uncovered a dual epithelial and mesenchymal phenotype of CTCs in the peripheral blood of metastatic carcinoma patients, still expressing epithelial markers such as cytokeratins but showing also expression of mesenchymal markers like Vimentin and the loss of E-cadherin (155). Furthermore, the majority of clusters of circulating tumor cells, circulating tumor microemboli (CTMs), show a higher fraction of Vimentin-expressing cells and also a higher resistance to apoptosis than isolated CTCs (156). The expression of these EMT markers were observed with high intra- and inter-patient heterogeneity, and the phenomenon of this partial or incomplete EMT in CTCs/CTM and their metastatic potential needs further detailed analysis.

1.4.7 EMT in cancer resistance to cell death

Although the involvement of EMT in metastasis is of great interest, its contribution to other incidences of tumor progression may also be relevant. Different cell lines have been found to escape apoptosis while undergoing EMT using this dedifferentiation process as a survival mechanism. For example in activated Ras-expressing mammary epithelial cells as well as in hepatocytes, treatment with TGFβ leads to inhibited apoptosis and induction of EMT (157). TGFβ as a potent promoter of tumor progression and invasion is further discussed in chapter 1.5. Twist, another EMT inducer, was found to activate survival by suppressing the Myc-mediated proapoptotic effect in neuroblastoma (158). Furthermore, Twist1 and Twist2 can also prevent oncogene-induced senescence via p16/ink4 and p21/cip inhibition (159). These data indicate, that EMT is not only an approach for tumor cells to maintain a mesenchymal invasive phenotype but also to ensure the survival of micrometastatic cells by circumventing premature senescence and apoptosis, two safeguard mechanisms against cancer.

1.4.8 EMT induces resistance to chemotherapy and immunosuppression

Several *in vitro* studies indicate coherence between EMT and resistance against chemotherapeutic therapies. For example, epithelial cell lines derived from colon carcinoma

or ovarian carcinoma, which are resistant to oxaliplatin or paclitaxel were shown to associate with the loss of epithelial markers and gain of mesenchymal morphology (160, 161). Moreover, depletion of Twist in breast cancer cells can partially reverse multidrug resistance (162). Consistent with these results, forced expression of the EMT-suppressing miR-200c was able to restore sensitivity to microtubule-targeting chemotherapeutic agents via its target gene class III β-tubulin (TUBB3) (163). Another drawback that cancer cells need to overcome is immune surveillance, and EMT is an effective approach to escape this safeguard mechanism, as shown in Neu-driven tumor mice, where tumor cells that have undergone immunoediting and lost antigen expression, upregulated genes associated with EMT (164). Moreover, after transgene deactivation in a Neu/ErBb2-inducible transgenic mouse model, tumor relapse from residual foci resulted in more aggressive new tumors of a mesenchymal phenotype, indicating that EMT offers a way to escape dependence on initial oncogenic pathways as well as resistance to targeted therapies (165). Interestingly, recent findings identified Snail as an activator of immunosuppressive cytokines, cytotoxic T lymphocyte resistance and the generation of impaired dendritic cells (166). This impairment of multiple immunosuppression and immunoresistance mechanisms leads to an altered response of host versus tumor, thus therapies targeting EMT inducers might not only interfere with metastasis but also retain immunocompetence in patients (149).

1.5 TGFβ and its role in cancer

The transforming growth factor (TGFβ) signaling pathway plays a dominant role in development, repair and homeostasis of most tissues in metazoan organisms. It belongs to a superfamily of growth factors consisting of more than 35 structurally related secreted proteins including also activins and bone morphogenic proteins (BMPs). These proteins regulate tissue differentiation by diverse cellular activities, such as proliferation, differentiation, migration, adhesion, ECM deposition and cell death (167). Because of these pleiotropic functions of TGFβ, a misregulation of this regulatory cytokine pathway is implicated in pathological conditions and can result in tumor development as discussed further in this chapter.

1.5.1 TGFβ signaling

The cytokine TGFβ is a secreted protein, existing in three variant forms (TGFβ1, β2 and β3). The inactive precursor form is bound in the extracellular matrix by the latent TGFβ binding proteins (LTBPs) and activated by proteolytic cleavage from the latent complex by convertase enzymes, such as furins (168). Bioactive TGFβ signals by forming a hetero-tetrameric receptor complex with two pairs of receptor serine/threonine kinases, namely TGFβRI and TGFβRII. Upon ligand binding, the type II receptors activate the TGFβRI by specific phosphorylation of serine and threonine residues in the juxtamembrane region, leading to subsequent conformational changes of the catalytic domain. The activated type I receptor then propagates the signal by phosphorylating associated effector proteins, such as the receptor-associated Smad proteins (R-Smads). Once activated, the R-Smads interact with the common mediator Smad4 and shuttle to the nucleus to control the transcription of various genes (169). Besides this well characterized canonical signaling pathway, TGFβ signals also via non-canonical Smad-independent signaling branches by interaction with various partners, such as receptor tyrosine kinases (RTKs) and diverse adaptor proteins. These two signaling branches, the canonical and the non-canonical pathways, can act independently from each other, but in most cases cooperate during TGFβ signaling. This complexity and flexibility of the TGFβ-induced signaling enables the comprehensive regulation of diverse cellular processes.

1.5.2 Canonical TGFβ signaling

The canonical TGFβ signaling activates Smad proteins to submit signals from the heteromeric receptor complex to the nucleus (Figure 4). The eight members of the mammalian Smad family can be divided in three groups according to their function: receptor activated Smads (R-Smads), consisting of Smad1, 2, 3, 5 and 8, the common-mediator Smad (Co-Smad) is Smad4, and both Smad6 and 7 belong to the inhibitory Smad group (I-Smads). The R-Smads are ligand specific signal mediators, of which Smad1, 5, and 8 are activated by BMP, whereas Smad2 and Smad3 mediate signals downstream of TGFβ and activins. Once activated, these R-Smads associate with each other, forming a trimeric complex with the common mediator Smad4 via their MH2 domains, to traffic into the nucleus and interact with various transcriptional regulators and co-activators like the CREB-binding protein (CBP)

or p300 (170-172). While the R-Smads and Smad4 are ubiquitously expressed, the regulation of the canonical TGFβ signal transduction pathway is controlled by an intrinsic negative feedback loop, exerted by three types of proteins, namely the inhibitory Smads, E3 ubiquitin ligases of the Smurf family and phosphatases like the SH2 domain-containing inositol 5-phosphatase (SHIP). After ligand stimulation, the inhibitory Smads translocate from the nucleus to the cytoplasm, where they suppress R-Smad activation by competitively interacting with type I TGFβR to inhibit R-Smad phosphorylation and recruit phosphatases like SHIP, which dephosphorylate and inactivate the TGFβ-receptor complex (173). Moreover, Smad7 can bind and activate the ubiquitin ligases Smurf1 and Smurf2 and recruit them to the TGFβ-receptor complex, thus leading to its endocytosis and lysosomal degradation by ubiquitinylation (174).

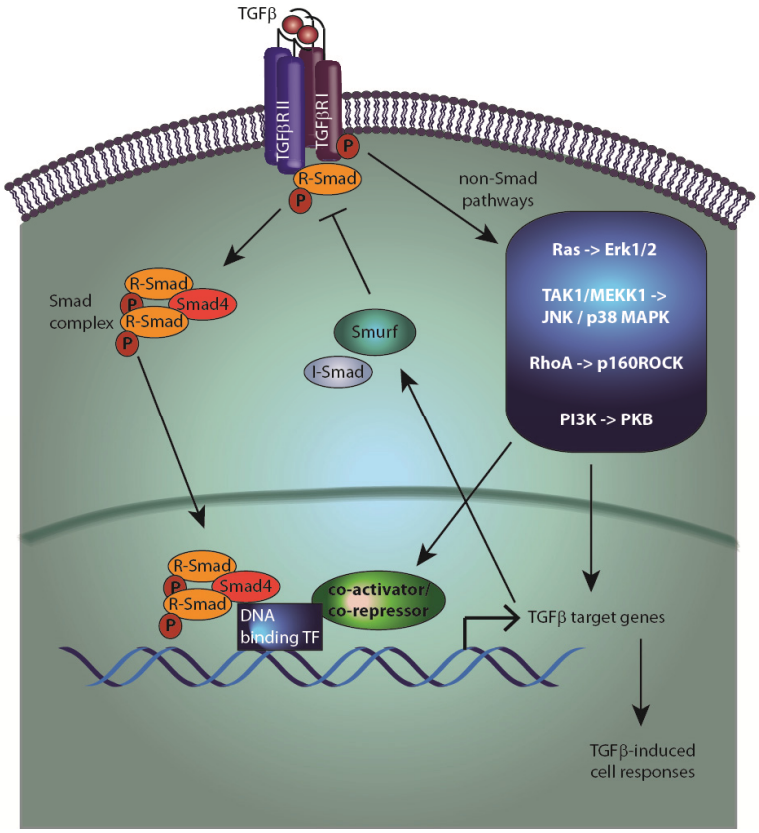


Figure 4: Intracellular signal transduction of TGFβ signaling. TGFβ signaling is transduced via the canonical and non-canonical pathways. TGFβ binds to its receptors leading to a transactivation of TGFβRI and subsequent phosphorylation of R-Smads (Smad2 and Smad3), which bind the common Smad, Smad4, and then translocate to the nucleus by forming a Smad complex. By interacting with co-activators and co-repressors and other transcritpion factors, the Smad complex regulates the transcription of TGFβ target genes. TGFβ stimulation also activates Smad independent signaling cascades, such as Ras-Erk, p38, JNK, PI3K-PKB, and small GTPases like RhoA.

1.5.3 Non-canonical TGFβ signaling

Besides Smad, the TGFβ-receptor complex interacts with proteins of various other signaling branches (Figure 4), including the small GTPase Ras, the mitogen activated protein kinases (MAPKs) Erks, p38 and c-Jun N-terminal kinases (JNKs) (175, 176). Each of these interaction partners can be activated by TGFβ and deploy signaling cascades, which can act independently from or interfere with the canonical Smad-dependent signaling.

1.5.3.1 TGFβ signaling through MAPK pathways

Multicellular organisms possess three well-characterized subfamilies of MAPKs, involved in the control of various physiological processes. Among the MAPKs activated by TGFβ, are the extracellular signal-regulated kinases Erk1 and Erk2, JNK1 and 2 and the four isoforms of p38 (p38α-δ). Activation of the TGFβ-receptor complex by binding to its ligand leads to the trans- and autophosphorylation of the type I and II receptors on multiple sites. These phosphorylation sites can then be recognized by adaptor proteins, including growth factor receptor binding protein 2 (Grb2) and the Src homology domain 2 (Shc2). Subsequent single or contiguous activation of these adaptor proteins leads to the activation of Ras with ensuing activation of the MAPKs Erk1/2, which has been shown to be important for the disruption of cells adherens junctions, ECM modulation and to accompany with increased motility and endocytosis (85). Furthermore, Erk1/2 can phosphorylate Smad2 and Smad3 in their linker region and Erk1/2 cooperation with Smad3 has been found to induce the expression of connective tissue factor (CTGF), which is involved in metastases formation (177). Conversely, activated Erk1/2 can also negatively regulate Smad2 and 3 by inhibiting nuclear accumulation and reducing Smad-dependent transcription (178). Another interaction partner of the activated TGFβ-receptor complex is the interleukin-1 receptor-effector module, called IL1R-TRAF6-TAK1, which is activated by subsequent polyubiquitination, leading to the activation of the MAPKs JNK and p38 via MKK4 and MKK3/6 (179). This TRAF6-TAK1-JNK/p38 activation is known to cooperate with the canonical TGFβ pathway to induce apoptosis or EMT (180).

1.5.3.2 TGFβ-induced PI3K activation

Another pathway, which can be activated by TGFβ is the phosphatidylinositol-3-kinase (PI3K), as indicated by the phosphorylation of its downstream effector protein PKB

(181). This can be direct via the TGFβRII-associated regulatory subunit p85, but can also result from cross-activation of the EGF receptor (182). PKB phosphorylation leads to the subsequent activation of mammalian target of rapamycin (mTOR), and via phosphorylation of its target proteins S6K and 4E-BP1 it is a key regulator of protein synthesis, shown to be important in TGFβ-induced EMT. Furthermore, activated PKB can regulate the sensitivity to TGFβ-induced apoptosis by direct interaction with Smad3 and inhibition of the pro-apoptotic transcription factor FoxO (183, 184).

1.5.4 TGFβ-mediated apoptosis

TGFβ is able to limit cancer formation by inducing a vast number of proteins, involved in apoptotis. Among the well characterized downstream targets for pro-apoptotic functions of TGFβ are the growth arrest and DNA damage-inducible protein β (GADD45β), BH3-only factors, such as Bim, and the death-associated protein kinase (DAPK), which all converge in the mitochondrial pathway with subsequent cytochrome C release and Caspase-dependent apoptosis (185-187). In breast cancer cell lines, Bim was found to be strongly induced by TGFβ in a FoxC1-dependent manner (188). In hepatocyte cells lines a conjoint upregulation of the two BH3-only proteins Bmf and Bim by TGFβ is essential to induce apoptosis (189). The expression of Noxa, another BH3-only protein, has been shown to be strongly upregulated by Notch signaling, which is known to synergistically regulate with TGFβ their common target genes via Smad3 in many cell types (190). These findings suggest, that the BH3-only protein family members play at least partially redundant roles in the regulation of TGFβ-induced cell death. Different mechanisms to promote cell death are used by activating the lipid phosphatase SHIP, which promotes apoptosis by inhibiting PI3K activity, thereby blocking its survival promoting signaling (191). Furthermore, interaction of Smad with PKB and TGFβR interactions with the p38 activator DAXX have also been proposed as mediators of apoptosis (168).

1.5.5 TGFβ mediates EGFR signaling

Focusing on the anti-apoptotic role of TGFβ in later stages of tumorigenesis opens a complex interplay of cellular signaling. In hepatocytes TGFβ has been shown to activate epidermal growth factor receptor (EGFR) and phosphorylate c-Src. Blocking of EGFR amplifies the apoptotic response to TGFβ1 without blocking the EMT process, indicating an

essential role of EGFR signaling in impairing TGFβ-mediated apoptosis (192). Furthermore, activation of EGFR is involved in the neoplastic transformation of solid tumors and its overexpression correlates with poor prognosis (193). It has been actually demonstrated that various epithelial tumors cells are significantly more sensitive to EGFR inhibition than tumor cells which have acquired mesenchymal phenotypes, including NSCLC, colorectal, head and neck and breast carcinomas (194-196). This data indicate, that the EMT status of a tumor can be a broadly applicable indicator of sensitivity to EGFR inhibitor therapies in patients.

1.5.6 The dual role of TGFβ

Perturbations of TGFβ signaling are frequently involved in tumor initiation and tumor progression through their effects on cellular processes. The growth inhibitory effect of TGFβ signaling in epithelial cells as well as the absence or decrease of the downstream Smad4 expression in various cancer types, including pancreatic, colorectal and head and neck cancers, establishes the role of TGFβ as a tumor suppressor (197, 198). However, during tumor progression, malignantly transformed cells frequently lose the responsiveness to or even distort the tumor suppressive effects of TGFβ. The evasion of cancer cells from the anti-proliferative effect of TGFβ was found to be more than just breaking the signaling circuitry. By activating the cellular EMT program late-stage tumors are able to redirect TGFβ to act as an autocrine tumor-promoting factor by elevating tumor growth, evasion of immune surveillance and cancer cell dissemination and metastasis (168, 199). These two faces of TGFβ signaling can be observed in various transformed cell systems, namely Ras-expressing mammary epithelial cells were found to preferentially undergo EMT and inhibit apoptosis when treated with TGFβ (157). In addition, the untransformed murine mammary gland cells (NMuMG) exhibit upon longterm TGFβ treatment a sustained EMT with resistance to apoptosis reflecting the long term effect of chronic TGFβ exposure in epithelial tissues during fibrosis and in cancer cells (200). The mechanism behind this dual function of the cytokine is based on the disruption of the signaling and the context, in which this disruption occurs. Malignant cells can bypass the suppressive effects of TGFβ either by inactivation of core components of the signaling pathway, such as TGFβR through mutation concomitant with loss of heterocygosity (197). The alternative path comprises selective alterations of the tumor-suppressive arm of the pathway, which then also allows the cancer cells to freely

adopt the remaining TGFβ regulatory functions to their advantage for pro-tumorigenic purposes (168). In both cases, cancer cells can make use of the TGFβ-mediated modulations of the microenvironment by enforcing immune tolerance, shielding cancer cells from immune surveillance. Moreover, malfunction of TGFβ signaling in immune cells can induce chronic inflammation, leading to a tumor-friendly environment (201, 202). Secretion of TGFβ by the tumor itself attracts stromal cells, such as myofibroblasts, which have been shown to facilitate tumor development and invasion by producing MMPs and pro-angiogenic factors, such as CTGF and VEGF (203, 204). Upregulation of angiopoietin-like 4 (ANGPTL4), a vascular remodeling gene and downstream target of TGFβ, helps to disrupt endothelial cell-cell junctions and by this, facilitates transendothelial extravasation of cancer cells at secondary sites (205). Also in distant organs, TGFβ exerts an important function in enhancing colonization and metastasis outgrowth shown by positive phospho-Smad2 stains of multiple bone, lung, liver and brain metastases of breast cancer patients (206). These highly diverse functions of TGFβ and its dual role as tumor suppressor and tumor promoter make it obvious that therapeutic targeting of the TGFβ pathway should reconstitute balanced intact signaling, as a distortion of the concinnity could lead to the transformation of normal cells to cancer cells.

1.6 Cancer stem cells

Due to hyperproliferation combined with genetic instability, advanced tumors are usually showing a high grade of heterogeneity, containing regions with distinct stages of differentiation, proliferation, inflammation and invasiveness. Although still debated, evidence emerged in the last few years for the existence of a new subclass of neoplastic cells within tumors, namely cancer stem cells (CSCs) or alternatively cancer-initiating cells (CICs). This CSCs are defined by their self-renewing capacity and the ability to newly generate tumors when transplanted into host mice. They are often accompanied by the expression of cell surface stem cell markers, also expressed by normal stem cells of the tissue, which gave rise to the tumor (207, 208). Cancer stem cells were first identified in hematopoietic malignancies, and later they were also found to be implicated in solid tumors like breast carcinomas, glioblastomas, pancreas carcinomas and neuroectodermal tumors (207, 209-212). The origin of CSCs within solid tumors is still unclear and might be variable and

dependent on the type of tumor. Cancer stem cells can either develop from normal tissue stem cells by oncogenic transformation triggered by the tumor microenvironment or alternatively from partially differentiated tumor cells that have adopted more stem-like properties. The latter theory is supported by the finding that forced expression of the transcription factor Oct4, a master regulator and gatekeeper of self-renewal and pluripotency, in normal breast cells leads to the generation of stem-like cells with increased tumor initiation capacity (213). Additionally, there is an increasing number of data suggesting that cells, which undergo EMT, acquire stem cell like properties (214) (further discussed in chapter 1.6.2). Several parallels have been drawn between normal and cancer stem cells to have a better understanding of the innate biology of cancer stem cells. While the relative quiescence of normal stem cells is in discrepancy to the uncontrolled and high proliferation of cancer stem cells, the two cell types share several essential characteristics, such as self-renewal capacity, expression of high levels of ABC transporters, high mobility and resistance to anoikis (215). A number of studies have shown that Integrin expression is required for tumor initiation and hyperproliferation in various types of epithelial cell-derived cancers and it has also been demonstrated that this protein family plays an important role in migration and invasion during cancer progression (216, 217). β1-Integrin (CD29) as well as α6-Integrin (CD49f) have been shown to be highly expressed in stem cells of various organs and to regulate their homeostasis and self-renewal capacity in brain, skin and mammary gland (218-220). Following the theory that similar signaling pathways are at work in normal and cancer stem cells lead to the identification of several cancer cell populations with stem cell properties and high expression of Integrins (further discussed in 1.6.1). Recent studies revealed the high resistance of leukemic stem cells towards cytotoxic treatments and moreover, glioblastoma stem cells are less sensitive to radiation than their non tumorigenic counterparts indicating an important role of this subpopulation in drug and radiation resistance and concomitant cancer recurrence (221, 222). Based on this evidence targeting CSCs will be one of the future clinical focus to fight therapy resistant cancer and tumor relapse in patients.

1.6.1 Cancer stem cell markers

One major concern in assessing a therapeutic anti-cancer strategy is indeed the damage or eradication of normal stem cells when targeting cancer stem cells by using shared markers. However, if the CSC is a transformed cell of normal progenitor origin, the surface markers should also contain progenitor specific surface proteins. This would allow to specifically identify and target CSCs. Indeed extensive studies on specific CSC surface markers as prognostic or drugable targets are currently ongoing. Several combinations of surface markers have been published up to date to identify cancer stem cell subpopulations, and the expression levels and combination of these markers have been shown to be tissue and species-specific. Among the proposed markers are the hyaluronate receptor CD44, CD24, CD133, aldehyde dehydrogenase (ALDH), CD90, members of the ABC family, Sca-1, Lgr5, CD29 and CD49f, some of them being more specific than others, but none of them is sufficient by itself to identify CSCs with adequate sensitivity and specificity. The most prominent marker combination was defined by the isolation of CSCs from pleural effusions with advanced stages of breast cancer by sorting for the $CD44^{+}/CD24^{-/low}$ subpopulation, proving that these cells have increased tumor initiation capacity in limiting dilution experiments (207). This surface marker combination was also found in several *in vitro* cell systems of human breast cancer to be enriched for cancer stem cells, showing increased tumor forming capacity, increased invasive properties and resistance to radiation (223-225). However, there is increasing evidence that the appearance of this marker combination is not shared by different species and that there can be differences in the marker expression levels between human and mouse models. Recent data suggest that in mouse tumors and tumor cell lines, the CSC subpopulation can rather be defined by high expression of CD24 combined with the expression of other markers, such as β1-integrin (CD29) or α6-integrin (CD49f). Stingl et al. and Shackleton et al. have shown that mouse mammary gland cells with colony formation capabilities (MaCFC) are $CD24^{high}/CD49f^{low}$ and more differentiated than the $CD24^{high}CD49f^{high}$ mammary gland repopulating units (MRUs) and found a $CD24^{+}/CD29^{high}$ subpopulation to be enriched in mammary stem cells (226, 227). Similar findings were reported for the combination of $CD24^{high}$ and $Prominin\text{-}1^{-}$ (CD133), which showed a higher colony forming capacity compared to $CD24^{high}/CD133^{+}$ or $CD24^{low}/CD133^{-}$ (228). These markers, which have been shown to be specific for mouse mammary stem cells could also be

used to identify tumor subpopulations enriched for cancer stem cells. For example Brca1-mutant mouse cancer cell lines contain a $CD24^{+}/CD29^{+}$ as well as $CD24^{+}/CD49f^{+}$ subpopulation showing increased proliferation and colony formation *in vitro* and enhanced tumor initiation capacity *in vivo* (229). Moreover, isolated cells from p53-null mouse mammary gland tumors showed a $CD24^{high}/CD29^{high}$ subpopulation with increased mammosphere formation and tumor initiation ability, and in Brca1/p53-mediated breast tumors the $CD24^{med}/CD29^{high}$ population exhibited increased tumorigenicity and cisplatin resistance (230, 231). Furthermore, the above mentioned Prominin-1, a cell membrane glycoprotein hitherto used for the identification of endothelial progenitor cells, has been established as a reliable marker for the identification of stem-like tumor cells in glioblastoma with distinct specificity depending on the glycosylation status (211, 232). Additionally, the concomitant expression of CD133 and ALDH correlated with reduced disease-free and overall survival in ovarian cancer patients and isolation of a $CD133^{+}/ALDH^{+}$ subpopulation from ovarian cancer cell lines resulted in a CSC enriched cell population with highly increased tumor forming capacity (233). A detailed study for the expression for diverse cancer stem cell markers in breast cancers according to tumor subtype and histological stages in human, revealed a large discrepancy among the histological groups. For example $ALDH1^{+}$ cells were present to a greater excess in basal-like and $HER2^{+}$ tumors than in luminal ones and the before described $CD44^{+}/CD24^{-}$ population had a much higher incidence in basal-like than in $HER2^{+}$ tumors (234). This variety of markers depending on tumor type, subtype and histological stage and the absence of conservation between different species manifest the need of more detailed investigations to utilize these markers as effective therapeutic targets for sustained cancer cure and prohibition of tumor relapse. Nevertheless, there is a hint that the quite conserved expression of members of the integrin family in cancers and their implication in tumor onset and progression makes them definitely an interesting target candidate, which warrants further investigations.

1.6.2 EMT and cancer stem cells

There is substantial evidence suggesting an association of CSCs with a specific state of differentiation. Hence, EMT has been studied as a process to produce cancer stem cells due to its ability to convert cells from one state of differentiation to another. Normal stem cells

and cancer stem cells may share a mesenchymal phenotype, which increases their capability to preserve stemness and retain migratory properties. This is the case with embryonic stem cells grown on matrigel, which have been shown to adopt a mesenchymal phenotype by performing the cadherin switch and expression of Snail factors, Vimentin and Metalloproteinases but retaining their pluripotency by the expression of Oct-4 and Nanog (235). Furthermore, it has been demonstrated that overexpression of the EMT-inducing transcription factor Snail in trophoblast stem cells induces a metastable phenotype by epigenetic changes resulting in the loss of epithelial maintenance while preserving self-renewal and multipotency (236). Indeed, the induction of EMT in transformed mammary epithelial cells has been shown to generate populations with enriched CSC subsets with increased tumorigenicity, mammosphere formation capacity and cell surface marker expression (214, 237). Furthermore, it has been shown that exposure to TGFβ and tumor necrosis factor α (TNFα) induces EMT in breast tumor cells and generates cells with increased self-renewal capacity, greatly increased tumor-seeding and increased resistance to chemotherapeutics, which are all hallmarks of breast cancer stem cells (BCSC) (238). Conversely, fractionation of naturally occurring normal and neoplastic mammary epithelial cells with CSC surface markers showed attributes of mesenchymal transdifferentiation as well as expression of mesenchymal markers such as Vimentin and Fibronectin (214). Another example linking EMT and cancer stem cell formation was found in pancreatic cancer cells by the overexpression of FoxM1, which lead to a *bona fide* EMT by the activation of ZEB1, ZEB2 and Slug concomitant with the expression of the CSC surface markers CD44 and EpCAM and increased sphere forming capacity (239). Moreover, induction of the EMT-activator ZEB1 in pancreatic and colorectal cancer not only promotes tumor cell dissemination, but also represses expression of the stemness-inhibiting miR-203 and members of the miR-200 family, which target stem cell factors, such as Sox2 and Klf4. Thus indicating, that ZEB1 links EMT-induction and stemness-maintenance by suppressing stemness-inhibiting microRNAs (240). Above all, the coherence between EMT and cancer stem cells was also demonstrated in a more clinical related background by the finding that treatment of breast cancer cells with TGFβ and TNFα generates mesenchymal cells with BCSC phenotype associated with downregulation of claudins, indicating a shift to the claudin-low molecular subtype (238). The claudin-low breast cancer subtype has been characterized by the low to absent

expression of luminal differentiation markers, high enrichment in EMT phenotype to most closely resemble the mammary epithelial stem cell (241). Taken together, EMT and cancer stem cells appear to be closely networked and are responsible to a great extend for therapy resistance and tumor relapse constituting a stepping stone in cancer treatments, for which better understanding may lead to new therapeutic platforms.

1.7 Forkhead box transcription factors

The family of Forkhead box (Fox) genes are defined by a conserved DNA binding domain of a winged helix structure acting as transcription factors, which have been found to serve as key regulators in embryogenesis, signal transduction, maintenance of differentiated cell states and tumorigenesis (242). There are three families of Fox genes, namely the Foxc, Foxf and Foxl1, that are extensively expressed in mesodermal tissue and form paralogous clusters in the genome (243, 244). One of the best characterized members of this family is Foxc2, which has been implicated in the regulation of EMT by interacting with Smad proteins and to be a key player in metastasis (152, 245). Moreover, Foxc1 and Foxc2 have been shown to be highly expressed in the claudin-low metaplastic breast cancer subtypes, which is associated with EMT and cancer stemness and high expression correlates with poor survival of breast cancer patients (246, 247). Furthermore, the overexpression of Foxm1 in pancreatic cancer cells lead to the acquisition of an EMT phenotype via upregulation of ZEB1 and ZEB2 as well as stem cell-like characteristics (239). Another famous member of the forkhead family is the Foxo transcription factor, which acts as a tumor suppressor in various cancer types. Foxo actively promotes apoptosis in a mitochondria-independent and dependent manner by inducing the expression of death receptor ligands such as FasL, as well as Bcl-2 family members such as Bim and Bcl-X_L (248). This data indicate a key regulatory role of the Forkhead protein family in the regulation of EMT and tumor progression. In this context, a less established forkhead protein came into our interest, which is Foxf2 (also known as Freac-2 or Fkhl6), a widly expressed protein in various mesenchymal tissues, adjacent to the epithelium, including the alimentary, respiratory and urinary tracts, central nervous system and organs of special sense and limb buds (249).

1.7.1 Foxf2

Foxf2 was first identified as a transcriptional activator, containing a forkhead domain for nuclear localization and two independent C-terminal activation domains. Foxf2 was found to interact with TBP and TFIIB, two components of the general transcription complex binding a specific motif (Figure 5) (250, 251). In a prostate cell line, Foxf2 expression was shown to be epigenetically controlled via histone trimethylation by EZH2 (252). During mouse development of the palatal mesenchyme, expression of several Fox family genes, including Foxf1 and Foxf2, were found to be specifically regulated by the sonic hedgehog (Shh) and its binding partner Smo, which are also implicated in EMT induction and crosstalk with Notch, EGF/FGF and TGFβ signaling (253, 254). Indeed, TGFβ-induced EMT is one of the mechanisms strongly involved in regulating fusion of the palatal cleft, and Foxf2 is upregulated in the mesenchyme of the secondary palate (255, 256). Moreover, Foxf2$^{-/-}$ mutant mice die shortly after birth due to cleft palate and abnormal tongue development, indicating an essential role of Foxf2 in this EMT-associated developmental process (257). Furthermore, Foxf2 expression was detected in the mesenchyme of the lung and gut indicated to regulate the activation of canonical Wnt-pathway and secretion of ECM proteins and Bmp4, which inhibits the production of Wnt5a in the mesenchymal cells, thereby maintaining the epithelial-mesenchymal structure. Epithelial cells of Foxf2$^{-/-}$ mice show typical signs of depolarization, and the subcellular localization of adherens junctions, normally confined to lateral membranes, expand into the basal and apical membranes. Even though in some cells a complete depolarization was observed, they fail to undergo apoptosis (258, 259). More recently, Foxf2 has been shown to be one of the direct targets of miR-301, which promotes breast cancer proliferation, invasion and tumor growth (260). This data indicates an important role of Foxf2 in maintaining tissue homeostasis and preservation of the mesenchymal phenotype. A better understanding of the FoxF2 protein and its biology may thus provide new opportunities for developing effective therapeutic approaches to treat cancer.

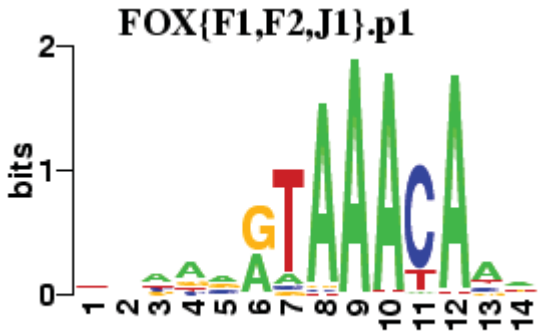


Figure 5: Weight matrix of the Foxf2 transcription factor binding motif. (kindly provided by Erik van Nimwegen)

2 Aim of the study

Epithelial to mesenchymal transition (EMT) is a complex process involved in cancer initiation and progression by modulating cell migration, cell differentiation and metastasis. Thus, identifying prognostic markers and/or therapeutic targets to block EMT-driven cancer cell invasion and circumvent drug resistance is essential for future cancer diagnostics and relapse-free therapy.

To gain new and more detailed insights into the molecular mechanism of EMT, we established different *in vitro* EMT model systems and analyzed changes in the gene expression profile to:

i. identify globally important regulators of EMT
ii. get insights into their biological function and their implication on EMT-associated acquirements such as increased resistance to apoptosis and enhanced cell migration and invasion.
iii. dissect the importance of EMT on the generation of cancer stem cells and characterize the stem cell properties of epithelial and mesenchymal cells

3 Materials and methods

Reagents and Antibodies

Recombinant human TGFβ (#240-B, R&D Systems), Small hairpin and silencing RNAs: shRNAs and siRNAs were purchased from Sigma-Aldrich, Control shRNA (shCtrl, Mission Non-Target shRNA control vector, #SHC002), murine Foxf2 shRNAs (MISSION® shRNA bacterial glycerol stock, shFoxf2 703 #TRCN0000084958, shFoxf2 704 #TRCN0000084960), murine Betacellulin siRNA (MISSION® siRNA, siBtc, #SASI_Mm02_00311942), murine Epiregulin siRNA (MISSION® siRNA, siEreg, #SASI_Mm01_00072956), murine Amphiregulin siRNA (MISSION® siRNA, siAreg, #SASI_Mm02_00316835), murine Noxa siRNA (MISSION® siRNA, siNoxa1,3 #SASI_Mm01_00077286,8), murine N-cadherin siRNA were purchased from Invitrogen (Stealth™ RNAi, siNcad4 #MSS202874, siNcad5 #MSS202875, siNcad6 #MSS202876). Antibodies: E-cadherin for immunofluorescence (#13-1900, Zymed), for western blot (#610182, Transduction Laboratories), N-cadherin for immunofluorescence (#610921, Transduction Laboratories), for western blot (#M142, Takara), NCAM (OB11, #9672, Sigma-Aldrich), Vimentin (#V2258, Sigma-Aldrich), ZO-1 (#617300, Zymed), Paxillin (#P13520, Transduction Laboratories), Fibronnectin (#F-3648, Sigma-Aldrich), PARP (#9542, Cell Signaling), EGFR (#2232, Cell Signaling), pEGFR (Y1173) (#sc-12351, Santa Cruz), cleaved Caspase 3 (#9664, Cell Signaling), Tubulin (#T-9026, Sigma-Aldrich), Actin (#sc-1616, Santa Cruz), GAPDH (#G8795, Sigma-Aldrich), HA-tag for ChIP (#ab9110, Abcam). Inhibitors: EGFR inhibitor AG1478 (#ALX-270-036, Alexis Biochemicals).

Cell culture and cell lines

All reagents used for cell culture were obtained from Sigma/Fluka (Basel, Switzerland) if not otherwise mentioned. All cells were cultured at 37 °C with 5 % CO_2 in DMEM medium supplemented with glutamine [2 mM], penicillin (100 U), streptomycin [0.2 mg/L] and 10% FBS. The subclone NMuMG/E9 (hereafter NMuMG) is expressing E-cadherin and has previously been described (261). MTdeltaEcad and MCF7-shEcad have been described (262). NMuMG-shSmad4 and NMuMG-shCont were obtained from P. ten Dijke (Leiden University Medical Center, The Netherlands; (263). NMuMG cells were treated with

TGFβ [2 ng/ml] without serum deprivation and TGFβ was replenished every 2 days. SiRNA transfections with LipofectAMINE RNAiMAX (Invitrogen) were performed according to the manufacturer's protocol 24h before treatment with TGFβ. The established cell line βT2 is derived from Rip1Tag2 induced pancreatic β-cell tumors and has previously been described (133). The established cell line Py2T is derived from MMTV-PyMT (264) mammary tumors (L. Waldmeier, unpublished data)

Generation of lentivirus

A cDNA encoding Foxf2 (kindly provided by Leif Lundh, Goteborg University, Sweden) (265) was tagged N-terminally with HA-tag and cloned into the lentiviral expression vector pLenti-CMV-Puro (kindly provided by Matthias Kaeser, Bern). Lentiviral particles were produced by transfecting HEK293T cells with the lentiviral expression vectors in combination with the packaging vector pR8.91 and the envelope encoding vector pVSV using Fugene HD (Roche). After two days the virus-containing HEK293T supernatant was harvested, filtered (0.45 µm), supplemented with polybrene (8 ng/ml) and used for target cell infection. Infections were performed twice a day for two consecutive days.

Growth Curves

One day before t_0 1.6 x 10^4 NMuMG cells were seeded in triplicates onto 24-well plates and transfected with the indicated siRNA. After 24h the cells were treated with TGFβ and cell numbers were determined by use of a Neubauer counting chamber.

Migration assay

NMuMG cells (2 x 10^4 /well) pretreated for 18 days with TGFβ were seeded in DMEM, 2 % FBS and TGFβ into the upper chamber of a cell culture insert, pore size 8 µm (Falcon BD, Franklin Lakes, NJ). The lower chamber was filled with DMEM 20% FBS and TGFβ. After 16h incubation at 37 °C and 5 % CO_2 the cells that had traversed the membrane were fixed in 4 % paraformaldehyde / PBS (15 min at room temperature), stained with DAPI [0.5 µg/ml] and counted by use of a fluorescence microscope.

Quantitative RT-PCR

Total RNA was prepared using Tri Reagent (Sigma-Aldrich), reverse transcribed with ImProm-II Reverse Transcriptase (Promega) and transcription levels were quantified by use of SYBR-green PCR Mastermix (Eurogentec) in a real time PCR system (Step One Plus, Applied Biosystems). Human or mouse riboprotein L19 primers were used for normalization. PCR assays were performed in duplicates and the fold induction was calculated against control-treated cells using the comparative Ct method ($\Delta\Delta C_t$). Following primers were used:

murine RPL19

fwd: 5'-CTCGTTGCCGGAAAAACA-3', rev: 5'-TCATCCAGGTCACCTTCTCA-3'

human RPL19

fwd: 5'-GATGCCGGAAAAACACCTTG-3', rev: 5'-CAGGGCAGTGATCTCCTTCTG-3'

murine Foxf2

fwd: 5'-AGCAGAGCTACTTGCACCAGA-3', rev: 5'-GCAGTCCGACTGAGAGATCCT-3'

human Foxf2

fwd: 5'-AGCAGAGCTACTTGCACCAGA-3', rev: 5'-GCAGTCCCACTGAGAGGTCCT-3'

murine E-cadherin

fwd: 5'-CGACCCTGCCTCTGAATCC-3', rev: 5'-TACACGCTGGGAAACATGAGC-3'

murine ZEB1

fwd: 5'-GCCAGCAGTCATGATGAAAA-3', rev: 5'-TATCACAATACGGGCAGGTG-3'

murine ZEB2

fwd: 5'-GGAGGAAAAACGTGGTGAACTAT-3', rev: 5'-GCAATGTGAAGCTTGTCCTCTT-3'

murine Id2

fwd: 5'-ACTATCGTCAGCCTGCATCA-3', rev: 5'-AGCTCAGAAGGGAATTCAGATG-3'

murine Noxa

fwd: 5'-CAGATGCCTGGGAAGTCG-3', rev: 5'-TGAGCACACTCGTCCTTCAA-3'

murine Btc

fwd: 5'-ACCAATGGCTCTCTTTGTGG-3', rev: 5'-CCGAGAGAAGTGGGTTTTCA-3'

murine Ereg

fwd: 5'-TTGACGCTGCTTTGTCTAGG-3', rev: 5'-GGATCACGGTTGTGCTGAT-3'

murine Areg

fwd: 5'-AAGAAAACGGGACTGTGCAT-3', rev: 5'-GGCTTGGCAATGATTCAACT-3'

Immunoblotting

Cells were lysed for 20 min on ice in Ripa-buffer (150 mM NaCl, 2 mM MgCl, 2 mM $CaCl_2$, 0.5 % NaDOC, 1 % NP40, 0.1 % SDS, 10 % Glycerol, 50 mM Tris pH 8.0, 2 mM Na_3VO_4, 10 mM NaF, 1 mM DTT, and a 1:200 dilution of stock protease inhibitor cocktail for mammalian cells (Roche). Protein concentration was determined using DC™ Protein Assay (BioRad Laboratories). Equal amounts of protein were diluted in SDS-PAGE loading buffer (10 % glycerol, 2 % SDS, 65 mM Tris, 1 mg/100 ml Bromphenolblue, 1 % betamercaptoethanol) and resolved by SDS-PAGE. SDS-PAGE gels were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore) by semi-dry transfer, blocked with 5 % skim milk powder in Tris-buffered saline with 0.05 % Tween 20 (TBST) and incubated with the indicated antibodies. HRP conjugated antibodies were detected using enhanced chemiluminescence. In some cases immunoblots were also quantified using the Odyssey Imager (Li-Cor-Biotechnology) or ImageJ 1.4.3.67 (Broken Symmetry Software).

Immunofluorescence Experiments

shCtrl or shFoxf2 NMuMG cells were plated on glass coverslips and treated for the indicated times with TGFβ [2 ng/ml]. Cells were fixed using 4 % paraformaldehyde /PBS for 10 min and permeabilized with 0.2 % Triton X for 2 min at room temperature. Then cells were blocked using 3 % BSA, 0.01 % Triton in PBS for 1 h at room temperature. The cells were incubated with the indicated primary antibody for 1 h followed by incubation with the fluorochrome-labelled secondary antibody (Alexa Fluor®, Invitrogen) for 1 h at room temperature. Nuclei were stained with 6-diamidino-2-phenylindole [1 µg/ml] (Sigma-Aldrich)

for 10 min. The coverslips were mounted (Fluorescent mounting medium, Dako) on microscope slides and imaged by using a confocal microscope (Leica SP5).

Apoptosis assay

Cells were washed twice in ice cold PBS and resuspended in 1x Annexin V binding buffer (0.01 M HEPES, pH 7.4, 0.14 M NaCl, 2.5 mM $CaCl_2$,) at a concentration of $1x10^6$ cells/ml. 5 µl of Cy5 Annexin V was added to $1x\ 10^5$ cells and incubated for 15 min on ice in the dark. Stained cells were filtered through a 40 µm mesh and analyzed on a FACSCanto II using DIVA Software (Becton Dickinson). Cell debris and duplets were excluded by a combination of light scatter and forward scatter plus width.

Flow cytometric analysis

Cells were washed twice in PBS supplemented with 5% FBS and stained with directly fluorochrome-labeled or biotin-conjugated monoclonal atibodies against CD44-PE (#553143, BD Pharmingen), CD24-Biotin (#553260, BD Pharmingen), CD29-Alexa647 (#MCA2298A647, Serotec), CD49f-Biotin (#MCA699BT, Serotec), followed by an incubation with streptavidin-fluorochrome secondary molecules, streptavidin FITC (#11-4317-87, eBioscience), streptavidin APC (#17-4317-82, eBioscience), streptavidin PE (#12-4317-87, eBioscience). Stained cells were analyzed on a FACSCanto II using DIVA software (Becton Dickinson). Dead cells were excluded by a combination of PI fluorescence and light scatter.

Proliferation assay (BrdU incorporation)

Cells were incubated with 10 µM BrdU for 2 h at 37 °C and 5 % CO_2. Then cells were fixed in 70 % ice-cold Ethanol and lysed by incubating first with 2 N HCl, 0.5 % Triton X-100 for 30 min and secondly in 0.1 M $Na_2B_4O_7$, pH 8.5 for 2 min at room temperature. The Nuclei were washed with 0.5 % Tween-20, 1 % BSA/PBS and incubated with FITC labeled anti BrdU antibody (#347583, Beckton Dickinson) for 30 min at room temperature. Nuclei were stained for DNA content by incubating with 5 µg/ml PI for a minimum of 1 h at room temperature. Stained cells were filtered through a 40 µm mesh and analyzed on a FACSCanto II using DIVA Software (Becton Dickinson).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) experiments were performed as previously described (266). In brief, crosslinked chromatin was sonicated to receive an average fragment size of 500 bp. Starting with 100 µg of Chromatin and 5 µg of HA-tag antibody, 1µl of ChIP material or 1 µl of input material were used for quantitative RT-PCR, usinf specific primers covering the *noxa* gene promoter region from basepair -696 to -499, the *betacellulin* gene promoter region from basepair -450 to -253, the *epiregulin* gene promoter region from basepair -851 to -654, the *amphiregulin* gene region in exon 2 from basepair +1086 to 1210, and primers covering an intergenic region as control. The amplification efficiencies were normalized between the primer pairs.

Microarray processing and data analysis

RNA was isolated using Tri Reagent (Sigma-Aldrich) from NMuMG cells infected with control shRNA or shRNA specific for Foxf2 treated with TGFβ for 0, 1, 4, 7 and 10 days. RNA quality and quantity was evaluated using an Agilent 2100 Bioanalyzer (Agilent Technologies). The manufacturer's protocols for the GeneChip platform by Affimetrix were followed. Methods included synthesis of the first- and second-strand cDNA followed by synthesis of cRNA by *in vitro* transcription, subsequent synthesis of single-stranded cDNA, biotin labeling and fragmentation of cDNA and hybridization with the microarray slide (GeneChip® Mouse Gene 1.0 ST array), posthybridization washings and detection of the hybridized cDNAs using a streptavidin-coupled fluorescent dye. Hybridized Affimetrix GeneChips were scanned using an Affimetrix GeneChip 3000 scanner. Image generation and feature extraction were performed using Affimetrix GCOS Software, and quality control was performed using Affimetrix Expression Console Software. Raw microarray data were normalized with Robust Multi-Array (RMA) and analyzed using Partek® Genomics Suite Software (Partek Inc.). One-way analysis of variance (ANOVA) and asymptotic analysis were used to identify significantly differentially expressed genes. The gene ontology (GO) tool from Partek® Genomics Suite Software as well as the Ingenuity Pathway Analysis Software (IPA®, Ingenuity Systems, Inc.) were used for further analysis.

Mammosphere-formation

Mammosphere-formation experiments were performed as previously described by Dontu et al. with minor modifications (267, 268). In brief, cells were used as a single cell suspension at a starting concentration of $1x10^5$ cells/ml in DMEM/F12 medium supplemented with B27 (Invitrogen), glutamine [2 mM], penicillin (100 U), streptomycin [0.2 mg/L], heparin [1 U/ml], hEGF [20 ng/ml] and bFGF [20 ng/ml], seeded on ultra low attachment (ULA) plates (Corning). Cells were incubated at 37 °C and 5 % CO_2 and medium was replenished by 25 % every second day of culture. Mammospheres were passaged after 7 days of culturing by 3-4 rounds of Trypsin incubation in turns with pipetting up and down to reach a single cell suspension. Single cells were reseeded for M2 or M3 at a concentration of $5x10^4$ cells/ml and/or used for FACS analyses.

Survival and metastasis correlation analysis

Analyses of Foxf2 expression correlation with breast cancer patient survival or metastasis formation were performed using two distinct datasets of the Netherlands Cancer Institute (NKI295) (269) and of the Memorial Sloan-Kettering Cancer Center (MSKCC) NY, described in detail in (57). The NKI295 database contained of 288 tumors of early-stage breast cancer (stageI and stageII) with information on Foxf2 expression, overall survival (10y) and time to metastasis. Tumors were divided into two groups based on the relative expression of Foxf2 to the tumor pools (logFC = 0) and further stratified by ER status (N=68 ER^-, N=220 ER^+). The Minn database consisted of microarray expression data from 82 patients, with more advanced (T2-T4) mammary carcinomas analyzed for overall metastasis free survival, also subdivided into lung and bone metastasis free survival. Tumors were divided into two groups based on the median expression of Foxf2 and further stratified by LN status (N=54 LN^+, N=28 LN^-). Overall and metastasis-free survival was estimated by applying Kaplan-Meier survival analysis and Cox proportional hazards regression modeling with the R survival package 2.36-5 and R version 2.11.1 (www.r-project.org). The p-value of the likelihood-ratio test was used to assess the statistical significance between the different patient groups.

Statistical analysis

Statistical analysis and graphs were generated using the GraphPad Prism software (GraphPAd Software Inc, San Diego CA). All statistical analyses were performed as indicated by paired or unpaired two sided t-test.

4 Foxf2 - a protagonist acting on two parts in EMT

4.1 Results

4.1.1 Foxf2 expression increases during EMT

To identify genes, which are critical key players in the onset and regulation of the EMT process, a gene expression profiling of three independent *in vitro* EMT model systems before and after EMT was performed in our laboratory (270). The three EMT cell systems used were (i) MTflEcad cells, an MMTV-neu (271) mammary tumor-derived cell line, in which both E-cadherin alleles were flanked by LoxP recombination sites (272). The complete ablation of E-cadherin was achieved by expression of Cre-recombinase (MTΔEcad) (262). (ii) In the human breast cancer cell line MCF7, EMT was induced by downregulation of E-cadherin using stable expression of shRNA (262). (iii) The third system used was the murine mammary epithelial cell line NMuMG, in which EMT was induced upon treatment with TGFβ (273) (Figure 6A). Screening for genes that are commonly regulated in all three EMT systems, we identified the forkhead transcription factor Foxf2 as a commonly upregulated gene. The transcriptional up- regulation was verified by quantitative RT-PCR (MTΔEcad 70x, MCF7-shEcad 28x, NMuMG +TGFβ 5x). In NMuMG cells, Foxf2 is up regulated already after 1 day of treatment and remains high in expression with consecutive TGFβ application (Figure 6B). To assess whether Foxf2 is a target of canonical or non-canonical TGFβ signaling, we monitored Foxf2 expression in stable Smad4 knockdown cells (NMuMG-shSmad4) (263) treated with TGFβ. Foxf2 mRNA expression levels were significantly reduced in TGFβ-treated NMuMG-shSmad4 compared to control cells, indicating that Foxf2 is regulated via canonical Smad4-dependent TGFβ signaling (Figure 6C).

Figure 6 : Foxf2 expression is upregulated during EMT via the canonical Smad pathway

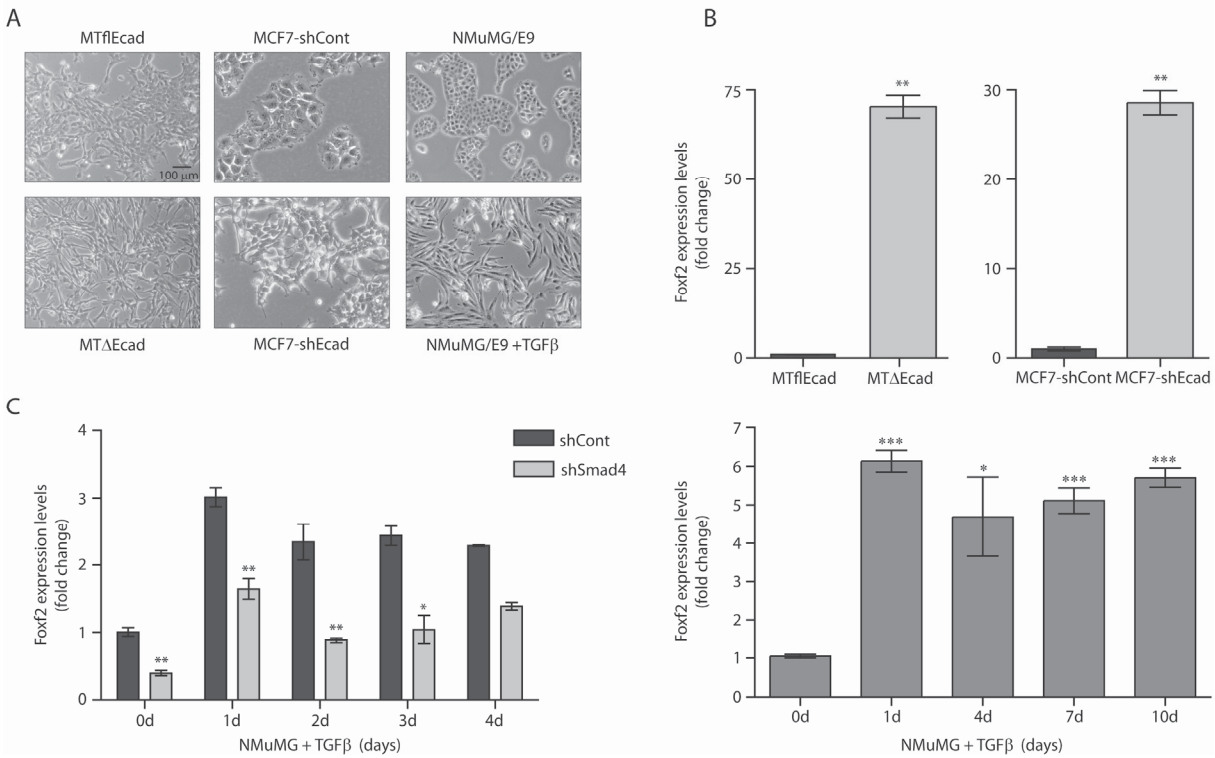
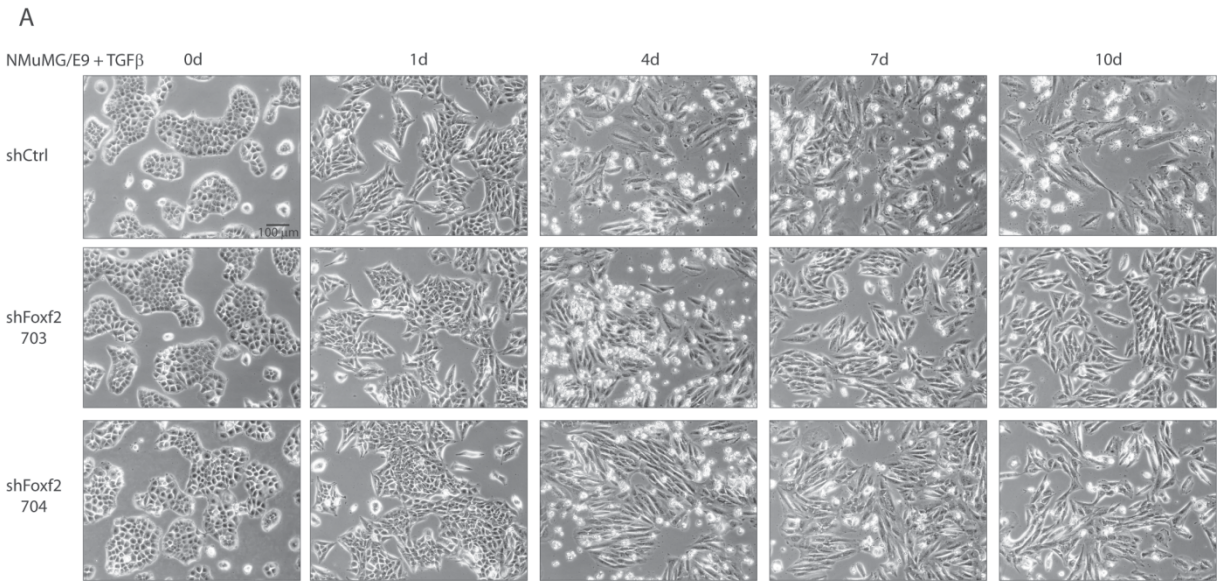


Figure 6: Foxf2 expression is upregulated during EMT via the canonical Smad pathway. (A) MTflEcad, MCF7 and NMuMG cells undergo EMT upon deletion of the E-cadherin gene by expression of Cre-recombinase (MTΔEcad), shRNA expression against E-cadherin (MCF7-shEcad) or treatment with TGFβ (NMuMG/E9+TGFβ) respectively. (B) Foxf2 mRNA levels were determined by quantitative RT-PCR in MTflEcad/MTΔEcad, MCF7-shCont/MCF7-shEcad, and NMuMG cells treated with TGFβ for the indicated times. Values were normalized to endogenous murine RPL19. (C) Foxf2 mRNA levels were quantified by quantitative RT-PCR in stable Smad4 knockdown (shSmad4) and control (shCont) NMuMG cells treated with TGFβ for the indicated times. Values were normalized to endogenous murine RPL19. Data are shown as mean of two independent experiments. Statistical values were calculated by using a paired two-tailed t-test. P-values ≤ 0.05 are indicated with (*), p-values ≤ 0.01 are indicated with (**), p-values ≤ 0.001 are indicated with (***).

4.1.2 Upregulation of Foxf2 is essential for complete EMT

To assess whether the expression of Foxf2 is able to induce EMT, NMuMG cells were stably infected with lentiviral particles encoding HA-tagged human Foxf2. Although the stable expression of Foxf2 in the nucleus of the cells could be demonstrated by immunofluorescence staining with anti-HA antibody, the cells did not gain an EMT-like phenotype (data not shown). To investigate whether the upregulation of Foxf2 is required for EMT we stably infected NMuMG cells with two different lentiviral particles encoding shRNA against murine Foxf2 (shFoxf2 703, shFoxf2 704). NMuMG-shFoxf2 cells treated with TGFβ show a clear change in morphology: they gain a spindle-like structure like the shCtrl cells. Yet, the NMuMG cells expressing shFoxf2 are not breaking down their tight cell-cell

contacts, which is a key step during EMT (Figure 7A). The knockdown efficiency of the shRNAs was determined by quantitative RT-PCR, showing a remaining upregulation when the cells were treated with TGFβ (Figure 7B), indicating that there could even be a stronger effect with complete abrogation of Foxf2 expression. To investigate whether the reduction of Foxf2 derogates the loss of epithelial markers and the gain of mesenchymal markers, we analyzed the expression of epithelial markers E-cadherin and ZO-1 and the mesenchymal markers N-cadherin, NCAM and Fibronectin. Western blot analysis displayed a sustained E-cadherin expression in Foxf2 knockdown cells even after 10 d of treatment with TGFβ, accompanied with a stabilization of ZO-1 expression. These results showed that the shFoxf2 cells did not show a *bona fide* EMT upon treatment with TGFβ, whereas Fibronectin, NCAM and N-cadherin expression remained unchanged (Figure 7C). By determining the mRNA expression levels of E-cadherin in Foxf2 knockdown and control cells we were able to show that E-cadherin expression is regulated at the transcriptional level by Foxf2 (Figure 7D). However, the mRNA expression levels of the mesenchymal markers NCAM and N-cadherin in shFoxf2 cells were comparable to the NMuMG control cells (data not shown).



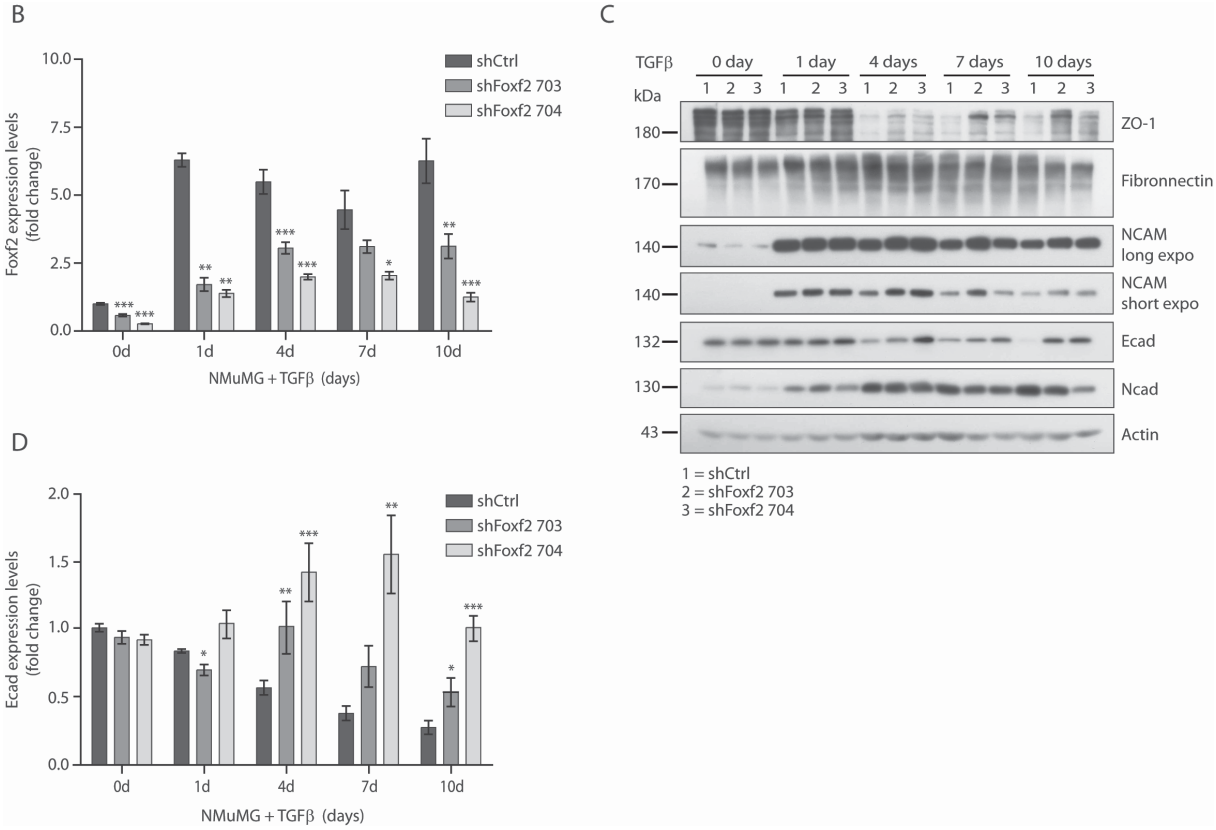


Figure 7: Downregulation of Foxf2 attenuates TGFβ-induced EMT. (A) Phase contrast micrographs of NMuMG cells stably expressing a control shRNA (shCtrl) or a Foxf2-specific shRNA (shFoxf2 703, shFoxf2 704) treated with TGFβ for the indicated times. (B) Knockdown efficiency was determined by quantitative RT-PCR for Foxf2 in NMuMG cells stably infected with shCtrl, shFoxf2 703 or shFoxf2 704, treated with TGFβ for times indicated. Values were normalized to endogenous murine RPL19. (C) Knocking down Foxf2 leads to a sustained expression of cell junction components. Immunoblotting analyses for the epithelial markers E-cadherin and ZO-1 as well as the mesenchymal markers NCAM, N-cadherin and Fibronectin in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the time indicated. Actin was used as a loading control. (D) E-cadherin downregulation during TGFβ-induced EMT is regulated at the transcriptional level by Foxf2. E-cadherin mRNA levels in shFoxf2 knockdown and control NMuMG cells were determined by quantitative RT-PCR. Values were normalized to endogenous murine RPL19. Data are shown as mean of three independent experiments. Statistical values were calculated by using a paired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***).

To investigate whether a downregulation of Foxf2 during EMT causes a difference in EMT-associated changes in cell adhesion, cell junctions and/or cytoskeletal composition, we performed an immunofluorescence staining for the cell adhesion proteins E-cadherin, N-cadherin and NCAM, the tight junction protein ZO-1, the focal adhesion protein Paxillin, Actin stress fibers (phalloidin) and the intermediate filament Vimentin. NMuMG shFoxf2 cells did not show a classical cadherin switch when treated with TGFβ. A normal upregulation of the mesenchymal marker N-cadherin was observed but E-cadherin was maintained at the cell membrane in contrast to the control cells, which show a *bona fide* EMT (Figure 8).

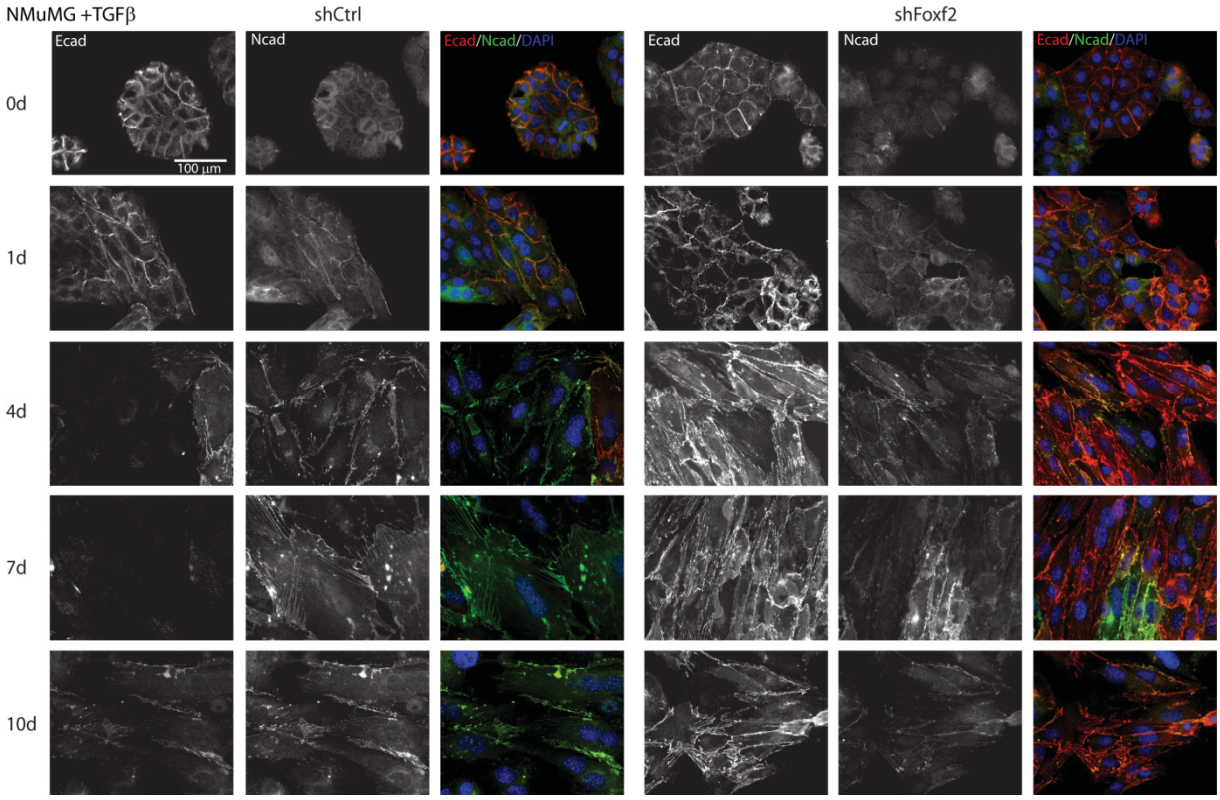


Figure 8: Foxf2 is required for TGFβ-induced disruption of adherens junctions. Representative confocal laser scanning microscopy analysis of E-cadherin (red) and N-cadherin (green) in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the indicated times. The nuclei are visualized by staining with DAPI (blue).

Furthermore we could also show that the stabilization of the cell adhesion protein E-cadherin is accompanied by a sustained localization of the tight junction component ZO-1 at the membrane, indicating that Foxf2 upregulation is required for cell junction disruption during TGFβ induced EMT (Figure 9).

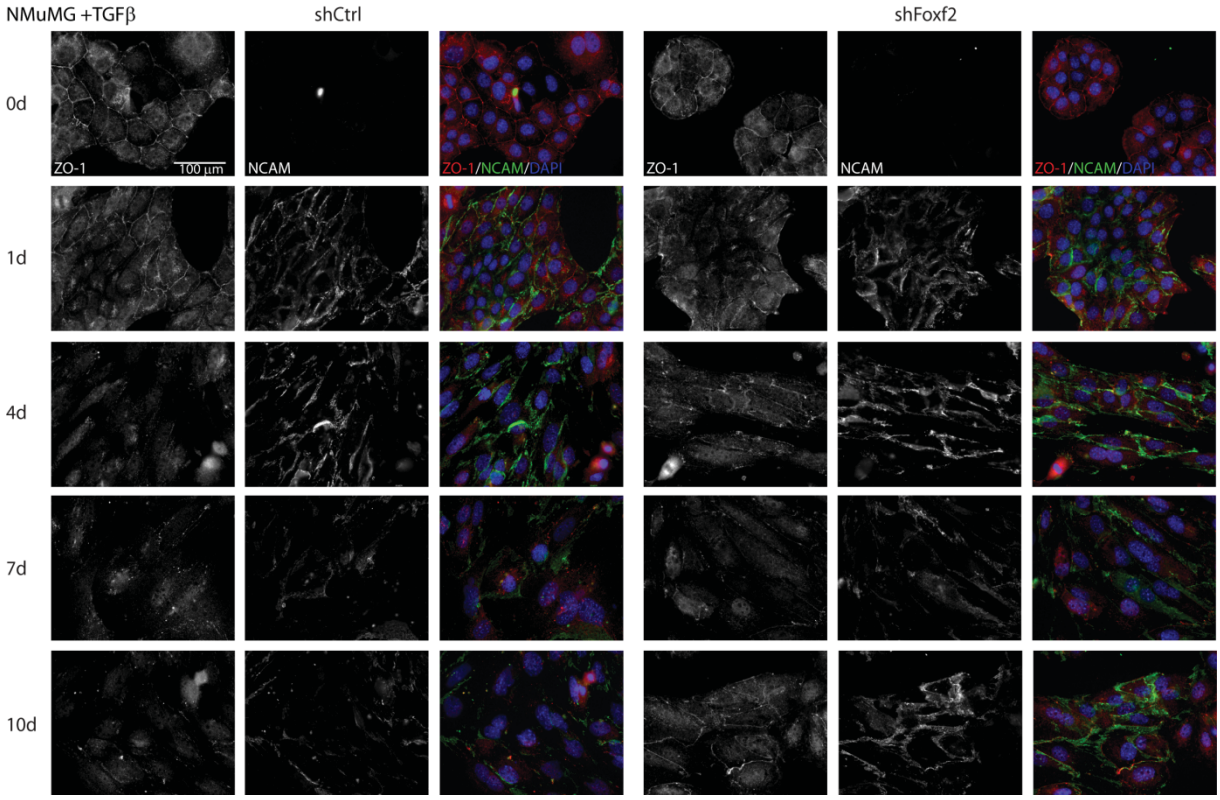


Figure 9: Reduced levels of Foxf2 expression results in a stabilization of tight junctions. Representative confocal laser scanning microscopy analysis of ZO-1 (red) and NCAM (green) in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the indicated times. The nuclei are visualized by staining with DAPI (blue).

Additionally, we observed that the upregulation of Foxf2 during EMT is not required for the EMT-associated cytoskeletal reorganization including the rearrangement of cortical Actin to stress fibers and the upregulation of filamentous Vimentin. Compared to the control cells, shFoxf2 knockdown cells showed a likewise upregulation of NCAM expression and a similar formation of focal adhesions shown by Paxillin staining (Figure 9,Figure 10Figure 11).

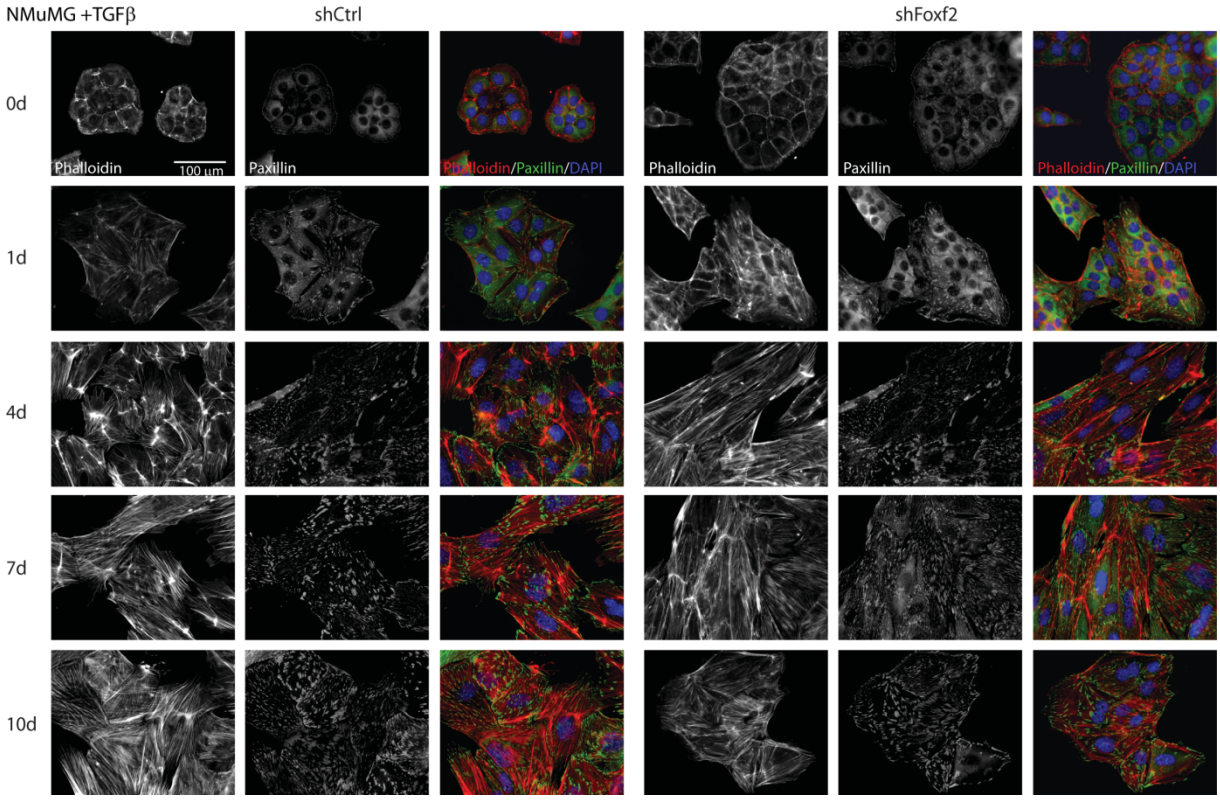


Figure 10: Foxf2 knockdown does not affect cytoskeletal rearrangement and focal adhesion formation of TGFβ-induced EMT. Representative confocal laser scanning microscopy analysis of filamentous actin (red) and paxillin (green) in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the indicated times. The nuclei are visualized by staining with DAPI (blue).

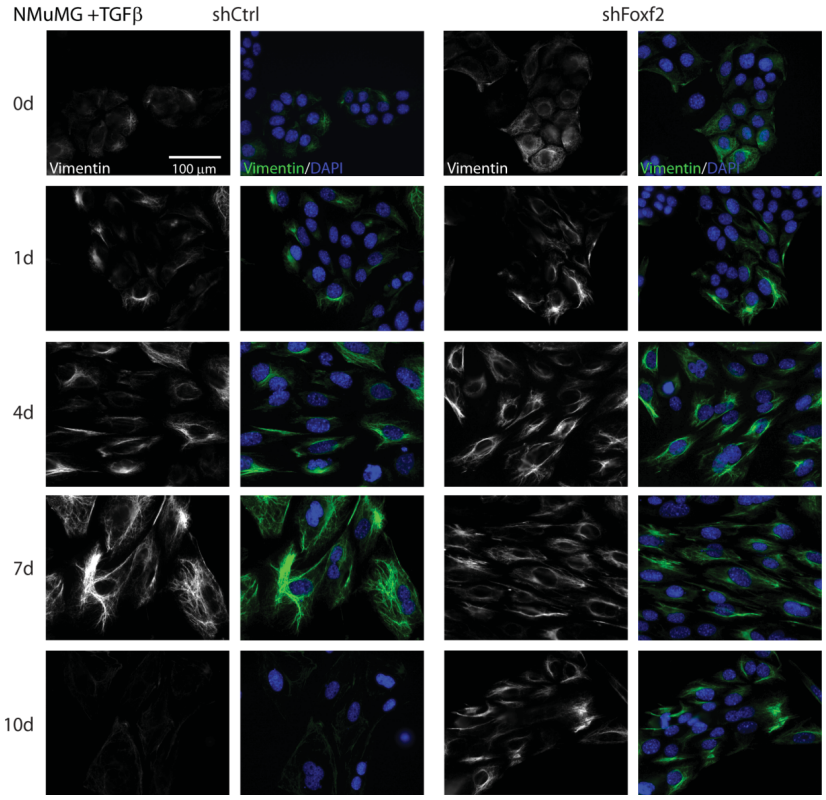


Figure 11: Foxf2 knockdown cells show EMT-associated upregulation of Vimentin to a similar extend as the NMuMG control cells. Representative confocal laser scanning microscopy analysis of Vimentin (green) in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the indicated times. The nuclei are visualized by staining with DAPI (blue).

4.1.3 Foxf2 regulates the expression of E-cadherin modulators

Although several genetic and epigenetic mechanisms have been identified, loss of E-cadherin is often attributed to transcriptional dysregulation. Several transcription factors were discovered as repressors of E-cadherin transcription. Among them the zinc-finger-homeodomain transcription factors ZEB1 and ZEB2 were found to interact in a ligand-dependent fashion with receptor-activated Smads involved in mediating TGFβ signaling (274) and to downregulate E-cadherin transcription by binding to the conserved E2-boxes in the *cdh1* promoter (275). By quantitative RT-PCR we have demonstrated that a downregulation of Foxf2 in TGFβ-treated NMuMG cells clearly attenuated the upregulation of the two transcription factors ZEB1 and ZEB2 (Figure 12A,B). These findings suspected that the regulation of E-cadherin by Foxf2 is at least partly mediated by the regulation of the ZEB proteins. Furthermore, levels of Id2 proteins were found to be dramatically decreased by TGFβ. Generally the Inhibitors of Differentiation (Ids) were found to act as positive

regulators of proliferation and as negative regulators of differentiation. The Id proteins lack a DNA binding motif and inhibit bHLH transcription factors, like HEB, E2-2 and E2A by binding with their HLH dimerization motif (276). By this mechanism, Id2 antagonizes E2A-dependent suppression of the E-cadherin promoter (277). Attenuation of Foxf2 in TGFβ treated NMuMG cells showed a significantly higher maintenance of Id2 expression compared to the control NMuMG cells (Figure 12C), which is consistent with the sustained expression levels of E-cadherin.

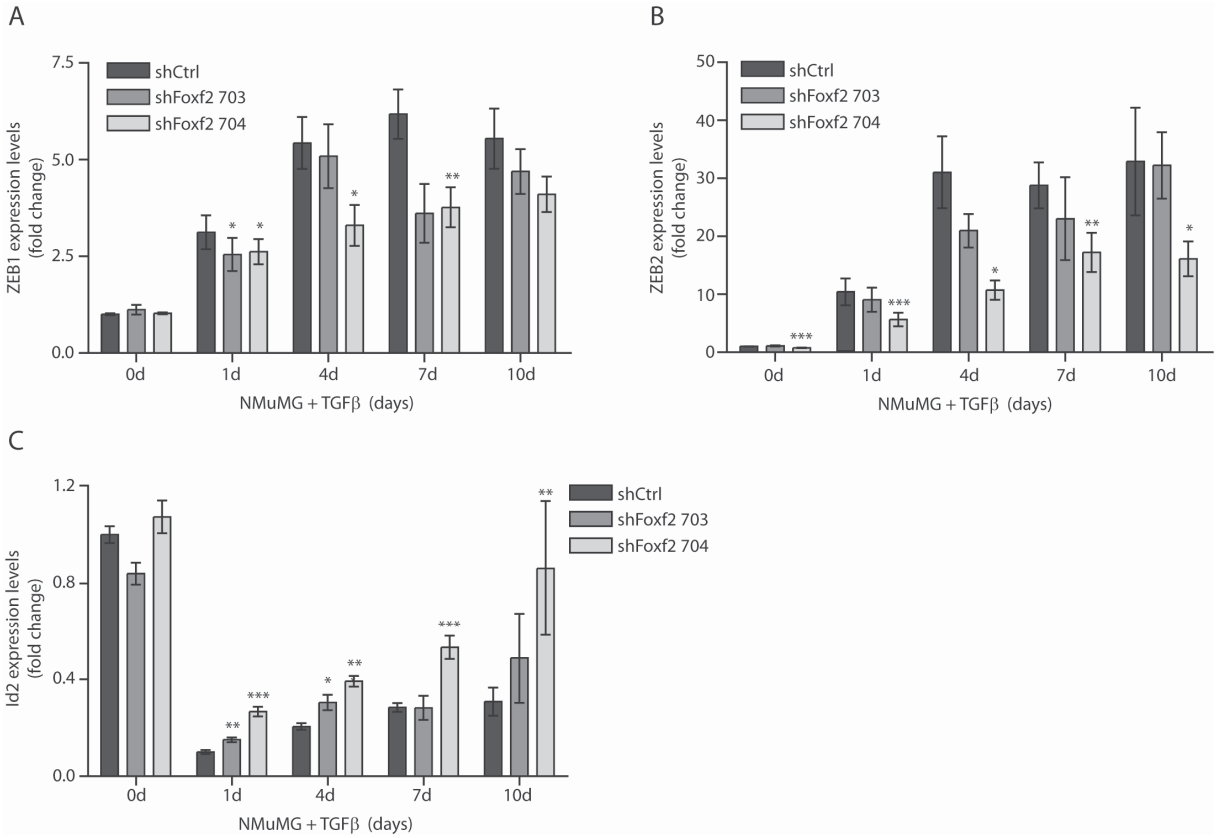


Figure 12: Foxf2 regulates the expression of E-cadherin modulators and transcriptional repressors. (A/B/C) ZEB1 mRNA levels, ZEB2 mRNA levels and Id2 mRNA levels, respectively, were determined by quantitative RT-PCR in shFoxf2 knockdown and control NMuMG cells. Values were normalized to endogenous murine RPL19. Data are shown as mean of three independent experiments. Statistical values were calculated by using a paired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***).

4.1.4 Downregulation of Foxf2 leads to reduced cell motility

An important initial step of fibroblast-like single cell migration is the disruption of intercellular contacts to leave the cell compound. This is accompanied by the loss of E-cadherin and acquisition of mesenchymal characteristics such as a spindle-shape, fibroblast

like morphology, which enables the cells to disseminate from their neighboring cells and facilitates their ability to migrate individually and invade (34). We have shown that the upregulation of Foxf2 is essential for the loss of E-cadherin and the disruption of cell junctions and therefore wanted to investigate whether cells deficient for Foxf2 were able to emigrate from the adjacent cells. We performed a migration assay comparing the migratory capability of TGFβ-treated, stable Foxf2 knockdown cells to the mesenchymal control NMuMG cells infected with control shRNA and found a decrease in motility for cells stably expressing a specific shRNA against murine Foxf2 (Figure 13A). Concomitant determination of the E-cadherin mRNA and protein expression levels revealed that the Foxf2 knockdown cells after 19 days of TGFβ treatment still express E-cadherin to a comparable extend of non treated epithelial NMuMG (Figure 13B,E). Although elongated, the cells deficient for Foxf2 show epithelial-like cell adherence when treated with TGFβ for 20 days contrary to their foxf2 expressing counterparts (Figure 13C). We were able to show that this effect is dose dependent, as there was a clear correlation between the efficiency of the Foxf2 knockdown and the expression levels of E-cadherin and the migratory capability (Figure 13D). In summary, the upregulation of Foxf2 in NMuMG cells undergoing EMT is essential for the transcriptional repression of E-cadherin and concomitant the disruption of the cell adhesions and the ability to migrate.

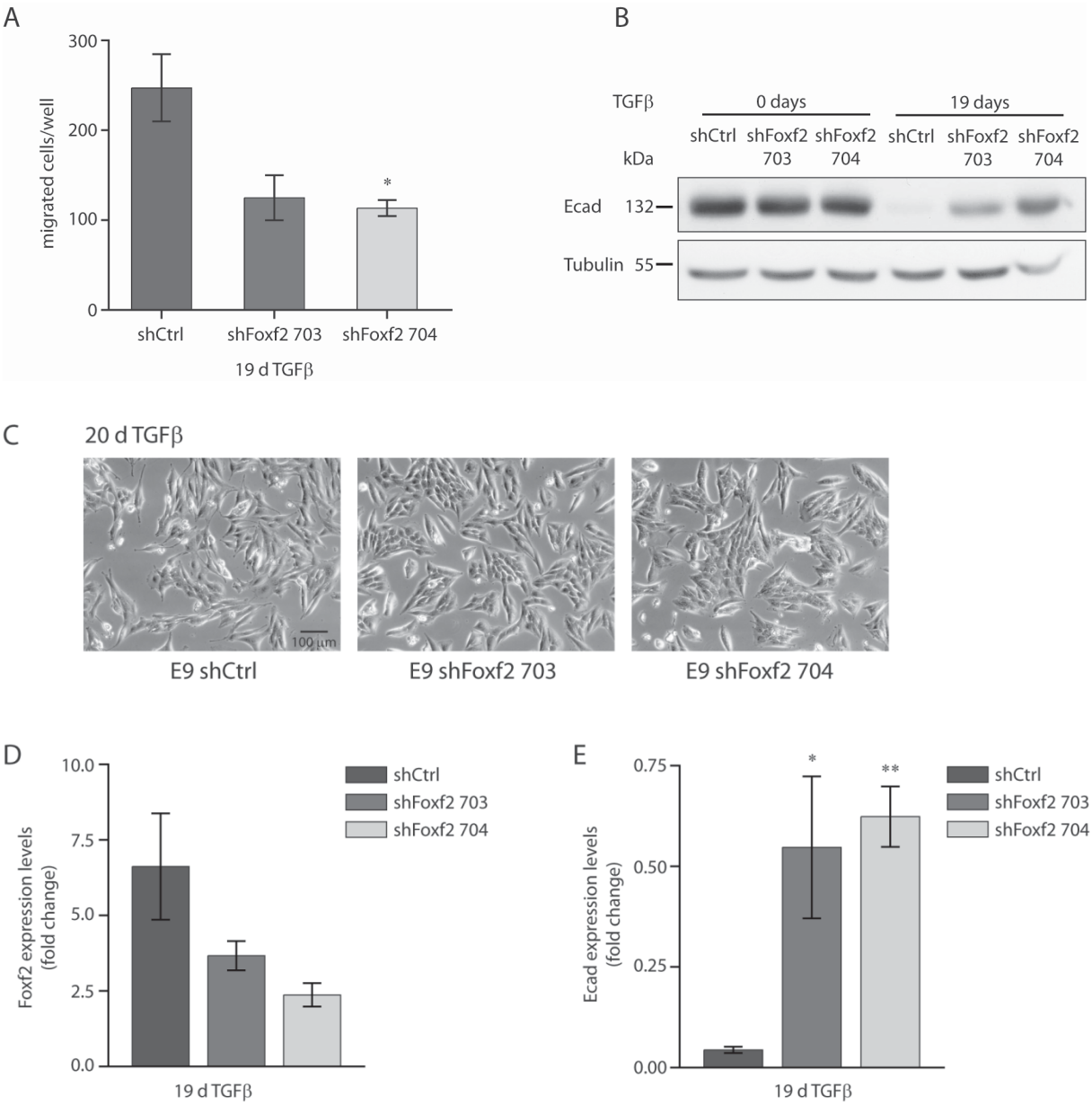
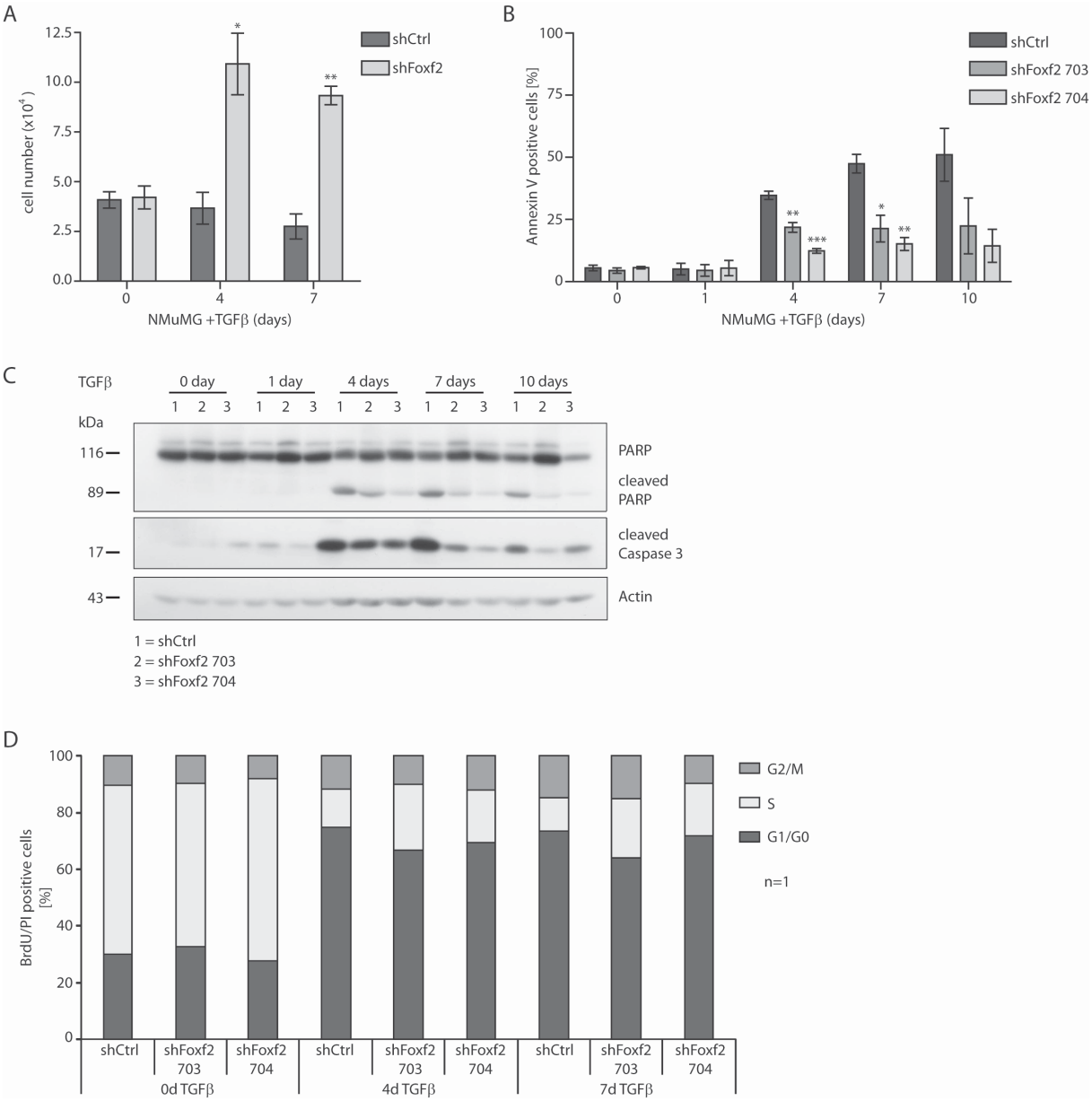


Figure 13: Depletion of Foxf2 expression reduces cell motility. (A) Depletion of Foxf2 leads to reduced cell migration of TGFβ-treated NMuMG cells through transwell filters compared to control cells (shCtrl). Data is shown as mean ±SEM of three independent experiments. Statistical values were calculated by using an unpaired two-tailed t-test. P-value ≤ 0.05 indicated with (*). (B) Knocking down Foxf2 leads to sustained E-cadherin expression after 19d of TGFβ treatment shown by immunoblotting analysis in shFoxf2 and shCtrl NMuMG cells. Tubulin was used as a loading control. (C) Phase contrast micrographs of NMuMG cells stably expressing a control shRNA (shCtrl) or a Foxf2 specific shRNA (shFoxf2 703, 704 respectively). (D/E) Foxf2 mRNA levels and E-cadherin mRNA levels respectively were determined by quantitative RT-PCR in shFoxf2 and shCtrl NMuMG cells treated for 19 d with TGFβ. Values were normalized to endogenous RPL19 and shown as fold induction to untreated shCtrl NMuMG cells. Data are shown as mean ±SEM of three independent experiments performed in parallel with the migration assay. Statistical values were calculated by using a paired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**).

4.1.5 Foxf2 is involved in TGFβ-induced apoptosis

We next investigated whether the depletion of Foxf2 has an impact on proliferation and/or cell death of NMuMG cells undergoing EMT. NMuMG cells stably expressing shRNA specific for Foxf2 showed TGFβ-mediated growth inhibition to a minor extent than the control cells (Figure 14A). To assess whether this is due to increased proliferation or decreased cell death, we compared the rates of apoptosis (Annexin V staining) and the rates of proliferation (BrdU incorporation and PI staining) of Foxf2 knockdown and control NMuMG cells treated with TGFβ. The depletion of Foxf2 significantly reduced the levels of apoptosis when compared to the control cells and only showed a minor difference in the number of cycling cells, indicating that the increased cell numbers observed can be attributed to an attenuation of TGFβ-induced apoptosis (Figure 14B, D, G). To investigate whether Foxf2 influences the Caspase-dependent programmed cell death we analyzed the activation of the zymogen Caspase 3 and its downstream cleavage target Poly (ADP-ribose) polymerase (PARP) by western blot analysis. Downregulation of Foxf2 shows an impairment of Caspase 3 and PARP cleavage in TGFβ-treated NMuMG cells (Figure 14C). In summary, we were able to show that the increase of Foxf2 is required for TGFβ induced cell death.


A
cell number (x10⁴)
12.5
10.0
7.5
5.0
2.5
0.0
0
4
7
NMuMG +TGFβ (days)
shCtrl
shFoxf2
*
**
B
Annexin V positive cells [%]
100
75
50
25
0
0
1
4
7
10
NMuMG +TGFβ (days)
shCtrl
shFoxf2 703
shFoxf2 704
**
***
*
**
C
TGFβ
0 day
1 day
4 days
7 days
10 days
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
kDa
116
89
17
43
PARP
cleaved PARP
cleaved Caspase 3
Actin
1 = shCtrl
2 = shFoxf2 703
3 = shFoxf2 704
D
BrdU/PI positive cells [%]
100
80
60
40
20
0
G2/M
S
G1/G0
n=1
shCtrl
shFoxf2 703
shFoxf2 704
0d TGFβ
4d TGFβ
7d TGFβ

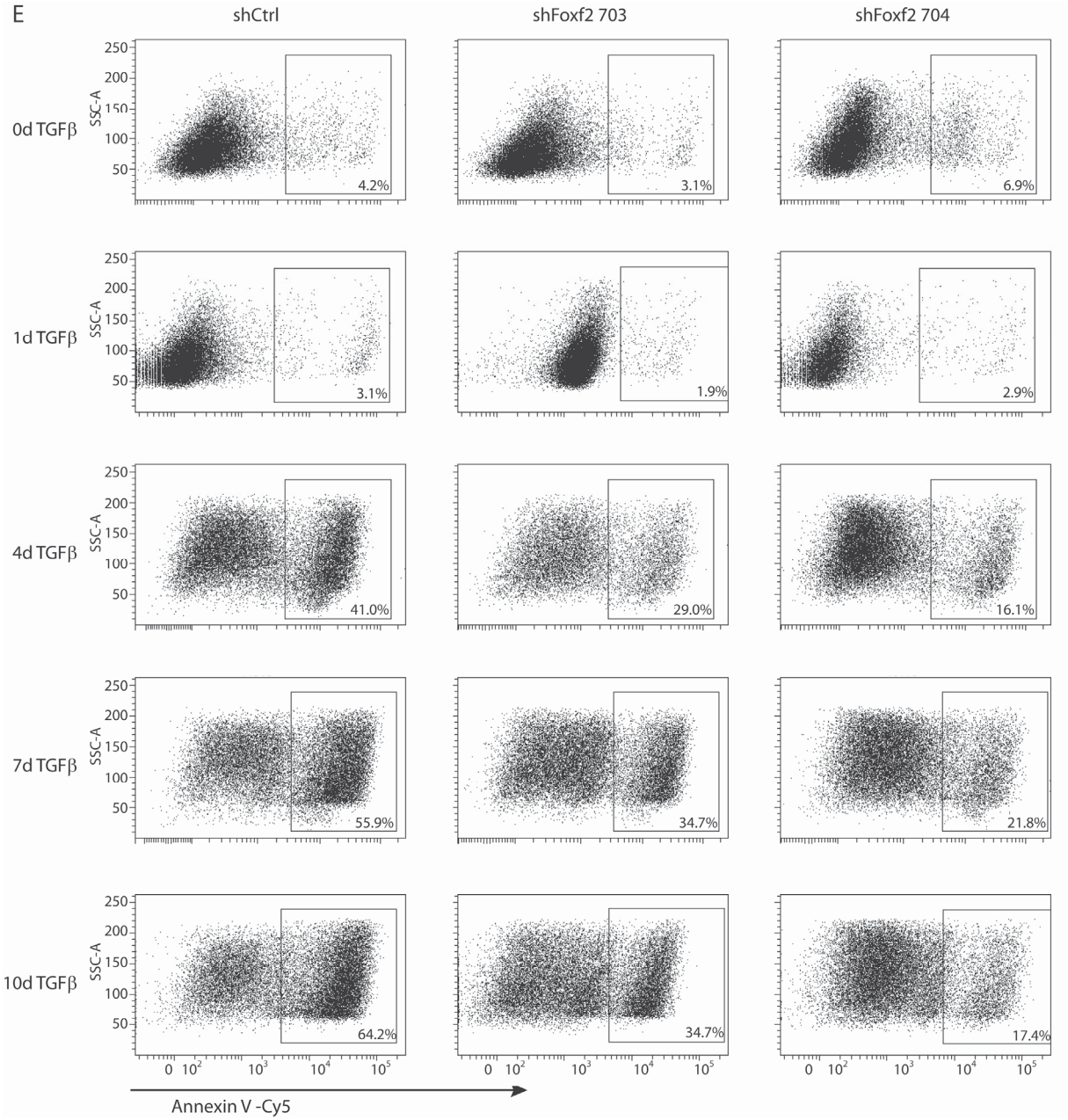


Figure 14: Depletion of Foxf2 attenuates TGFβ-induced apoptosis. (A) Downregulation of Foxf2 promotes accelerated cell growth. shFoxf2-expressing and control NMuMG cells were treated with TGFβ for the indicated times and counted using a Neubauer chamber. Representative result of three independent experiments. (B) Foxf2 depletion decreases the level of apoptosis during TGFβ induced EMT. shFoxf2 expressing and control NMuMG cells were treated with TGFβ for the indicated times and apoptosis was detected by Annexin V staining and FACS analysis. Data are means of three independent experiments. Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***). (C) TGFβ induces classical caspase-dependent apoptosis, and is regulated by the upregulation of Foxf2. Immunoblotting analyses for cleaved caspase 3 and PARP in shFoxf2 and control NMuMG cells treated with TGFβ for the times indicated. Actin was used as a loading control. (D) Foxf2 downregulation leads to a minor increase in proliferation. shFoxf2 cells and control NMuMG cells were treated with TGFβ for the times indicated and proliferation was assessed by BrdU incorporation and PI staining by FACS analysis. (E) shFoxf2 expressing and control NMuMG cells were treated with TGFβ for the indicated times and apoptosis was detected by Annexin V staining and FACS analysis. Data shown are representative results out of three independent experiments, qualified in panel B.

4.1.6 Foxf2 affects apoptosis by regulating the expression of Noxa

TGFβ has been shown to act as a tumor suppressor in early stages of tumorigenesis by inducing cell cycle arrest and apoptosis (278). Canonical TGFβ signaling results in the regulation of various apoptotic factors. To investigate which genes involved in apoptosis are regulated by Foxf2 we performed a gene expression profiling using GeneChip microarray (Affimetrix) on shFoxf2 and shCtrl NMuMG cells. Data analysis for differently expressed genes upon Foxf2 depletion during EMT using Partek Software revealed a regulation of Noxa by Foxf2. This expressional regulation could be confirmed by quantitative RT-PCR (Figure 15A). As the upregulation of Foxf2 is necessary for the increase in Noxa expression, we wanted to assess whether loss of Noxa is sufficient to prevent apoptosis in TGFβ-treated NMuMG cells. To address this question we knocked down Noxa by the use of two different siRNAs (siNoxa1, siNoxa3) to analyze apoptosis and cell growth. Consequently, following the reduction of Noxa mRNA levels (Figure 15B), apoptosis was attenuated significantly (Figure 15C) and cell growth inhibition was compensated (Figure 15D), indicating that depletion of Noxa is sufficient to preserve NMuMG cells from TGFβ induced apoptosis. Chromatin immunoprecipitation (ChIP) experiments with NMuMG cells expressing HA-tagged Foxf2 treated for 2 days with TGFβ demonstrated a direct binding of Foxf2 to the *noxa* gene promoter (Figure 15F).

Together these data indicate that Foxf2 is involved in TGFβ apoptosis by direct transcriptional activation of the pro-apoptotic protein Noxa.

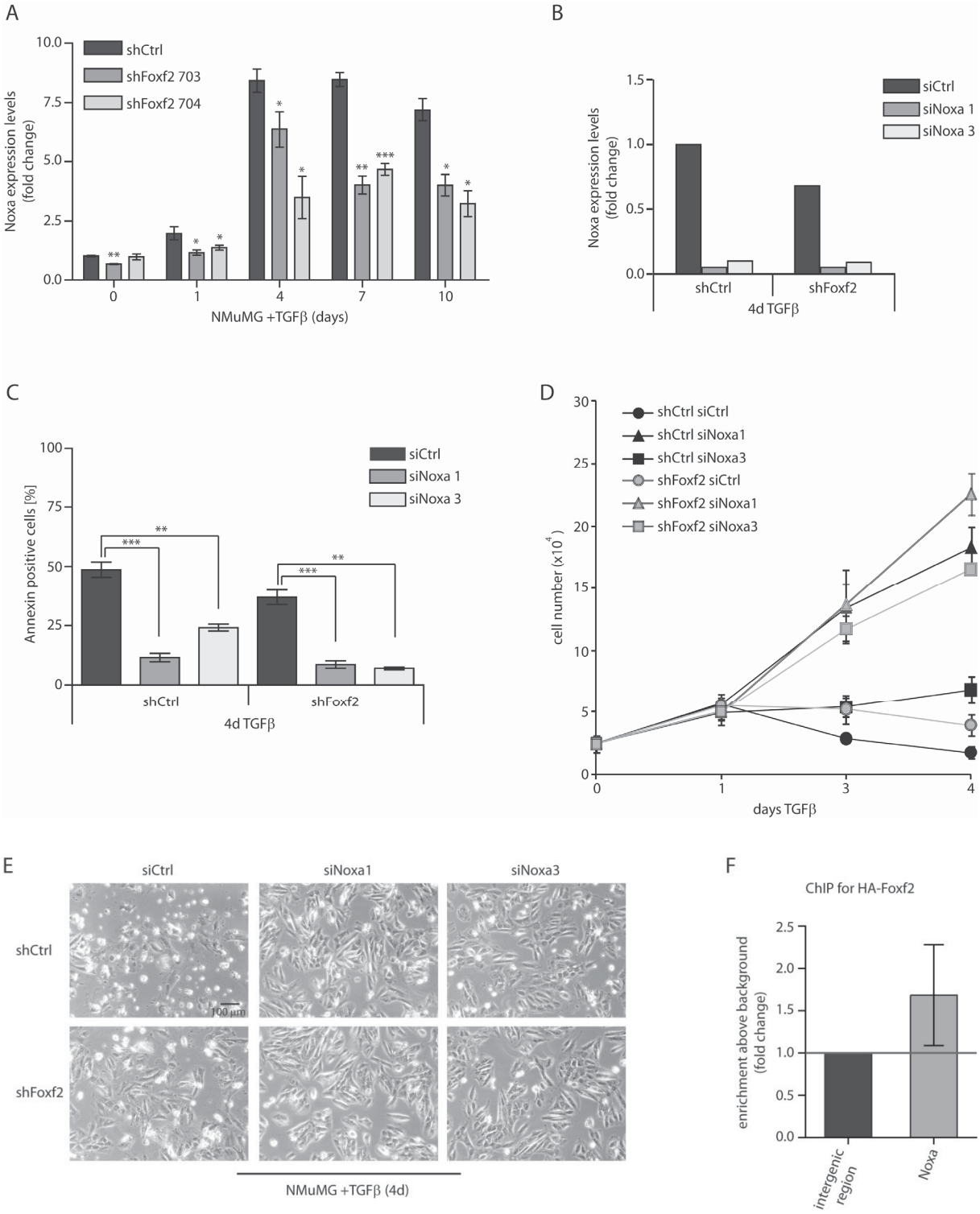


Figure 15: Noxa depletion prevents NMuMG cells from TGFβ induced apoptosis. (A) Knockdown of Foxf2 attenuates the upregulation of Noxa expression. Noxa mRNA levels in shFoxf2 and control NMuMG cells treated with TGFβ for the number of days indicated, were determined by quantitative RT-PCR. Values were normalized to endogenous murine RPL19. Data presented as mean ±SEM of three independent experiments. Statistical values were calculated using a paired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***). (B-E) shFoxf2 and control NMuMG cells were transfected with control siRNA (siCtrl) and two different siRNAs specific for murine Noxa (siNoxa1, siNoxa3) and incubated with TGFβ for the indicated times. (B) Noxa mRNA levels were determined by quantitative RT-PCR and values were normalized to endogenous RPL19. (C) Noxa depletion significantly decreases TGFβ induced apoptosis. Apoptosis was detected by Annexin V staining and FACS analysis. Results presented as

mean ± SEM of three independent experiments. Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***). (D) The impairment of Noxa leads to accelerated cell growth. Cell numbers were determined using a Neubauer chamber. (E) Phase contrast micrographs of shFoxf2 and control NMuMG cells transfected with siCtrl or siNoxa1/3. (F) Foxf2 regulates Noxa expression by direct transcriptional activation. Chromatin immunoprecipitaiton of HA-tag was performed either on Foxf2 expressing or control NMuMG cells treated for 2 days with TGFβ. Immunoprecipitated DNA fragments were quantified by quantitative PCR using primers covering basepairs -696 to -499 of the *noxa* promoter region and primers covering an intergenic region were used as negative control.

4.1.7 Foxf2 is involved in TGFβ-induced growth inhibition via EGFR inhibition

Besides directly activating the transcription of the *noxa* gene and inducing apoptosis we here demonstrate that Foxf2 also regulates EGFR signaling by transcriptional regulation of its ligands. EGFR family members are well known to protect from TGFβ-induced cell-cycle arrest and apoptosis by activating the PI3K pathway (192, 279). We were able to show that the survival signaling of activated EGFR is impaired by TGFβ in NMuMG cells and that this effect is dependent on the upregulation of Foxf2. Immunoblotting analyses revealed that the levels of activated (phosphorylated on Tyrosine 1173) forms of EGFR were higher in the stable shFoxf2 cells compared to the control NMuMG cells when treated with TGFβ (Figure 16A). To investigate how Foxf2 influences EGFR activation, we assessed whether the expression of members of the EGF family was regulated. Gene expression analysis and confirmation by quantitative RT-PCR revealed that the EGFR-ligands Betacellulin and Amphiregulin but not Epiregulin show sustained expression upon knockdown of Foxf2 (Figure 16B-D). By use of promoter binding prediction programs a direct binding of Foxf2 to the *betacellulin* promoter could be expected (data not shown). We were able to verify the direct binding of the transcription factor Foxf2 not only to the *betacellulin* promoter region but also to the regulatory region in exon 2 of the *amphiregulin* gene and to a smaller extent to the *epiregulin* promoter region by ChIP experiments on HA-tagged Foxf2 expressing NMuMG cells treated for two days with TGFβ (Figure 16E). In addition, the MAPK and PI3K pathways were described to be interactively engaged upon TGFβ signaling to assure survival and proliferation (280). Hence we investigated whether these pathways are involved in TGFβ-resistant growth of Foxf2 knockdown cells. As shown, Erk phosphorylation levels are increased in the control NMuMG cells upon TGFβ treatment whereas they remain constant in the NMuMG cells expressing shRNA specific for Foxf2, indicating that Foxf2 depleted cells do not activate the survival signaling via MAPK activation (Figure 16F). To investigate

whether PI3K pathway is regulated by Foxf2 to influence TGFβ resistant cell growth we assessed the phosphorylation of the downstream signaling kinase PKB. We observed a sustained phosphorylation on the activation site serine 473 in the Foxf2 depleted NMuMG cells (Figure 16G) compared to the control shRNA expressing cells.

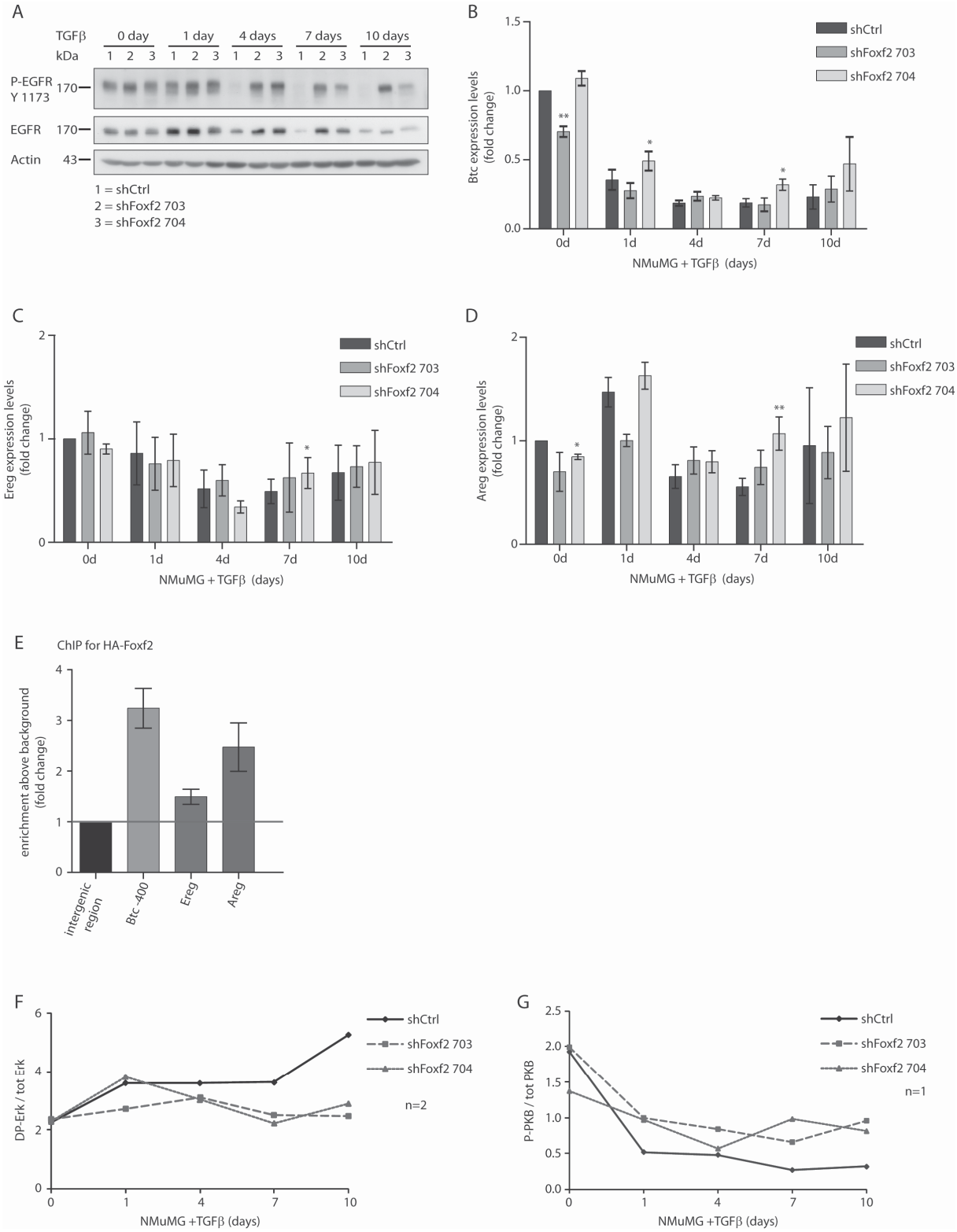


Figure 16: Foxf2 represses EGFR signaling via regulation of its ligands Betacellulin and Amphiregulin. (A) Depletion of Foxf2 leads to sustained EGFR activation. Representative immunoblotting analyses for the phosphorylation of EGFR and total EGFR protein levels in shFoxf2 knockdown and control NMuMG cells treated with TGFβ for the times indicated. Actin was used as a loading control. (B/C/D) Betacellulin, Epiregulin and Amphiregulin mRNA levels respectively were determined by quantitative RT-PCR in shFoxf2 and control NMuMG cells treated with TGFβ for the indicated times. Data are shown as mean ±SEM of three independent experiments. Statistical values were calculated by using a paired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**). (E) Foxf2 regulates the expression of EGFR ligands by direct transcriptional repression. Chromatin immunoprecipitaiton of HA-tag was performed either on Foxf2 expressing or control NMuMG cells treated for 2 days with TGFβ. Immunoprecipitated DNA fragments were quantified by quantitative PCR using primers covering basepairs -450 to -253 of the *betacellulin* promoter region, basepairs -851 to -654 of the *epiregulin* promoter, and basepairs +1086 to 1210 of the *amphiregulin* exon 2 and primers covering an intergenic region were used as negative control. (F) Foxf2 knockdown suppresses Erk activation. Representative immunoblotting analysis for the phosphorylation of Erk and total Erk protein levels in shFoxf2 and control NMuMG cells treated with TGFβ for the times indicated. Band intensities were quantified using the Odyssey Imager (Li-Cor-Biotechnology). (G) Depletion of Foxf2 leads to sustained PKB activation. Immunoblotting analysis for the phosphorylation of PKB and total PKB protein levels in shFoxf2 and control NMuMG cells treated with TGFβ for the times indicated. Signal intensity was quantified using ImageJ software (Broken Symmetry Software).

4.1.8 Inhibition of EGFR induces apoptosis in Foxf2 depleted NMuMG cells

As we were able to show that Foxf2 negatively regulates EGFR driven proliferation and survival during TGFβ-mediated EMT, we next investigated whether inhibition of EGFR signaling impairs the anti-apoptotic effect of Foxf2 depletion. For this objective we treated shFoxf2 and control NMuMG cells with the EGFR inhibitor AG 1478, which inhibits EGFR activation (Figure 17D), and assessed cell growth and apoptosis. Combined treatment with EGFR inhibitor (EGFRi) and TGFβ for four days lead to a significantly reduced growth of shFoxf2 expressing NMuMG cells compared to the solvent (DMSO) treated control (Figure 17B). In addition we could show that treating shFoxf2 expressing NMuMG cells with AG 1478 increases the apoptosis to a similar extent as in the control cells (Figure 17C), which correlates with the levels of EGFR inhibition (Figure 17D). This result indicated that TGFβ-resistant growth of Foxf2 knockdown cells relied also on the activation of EGFR survival signaling.

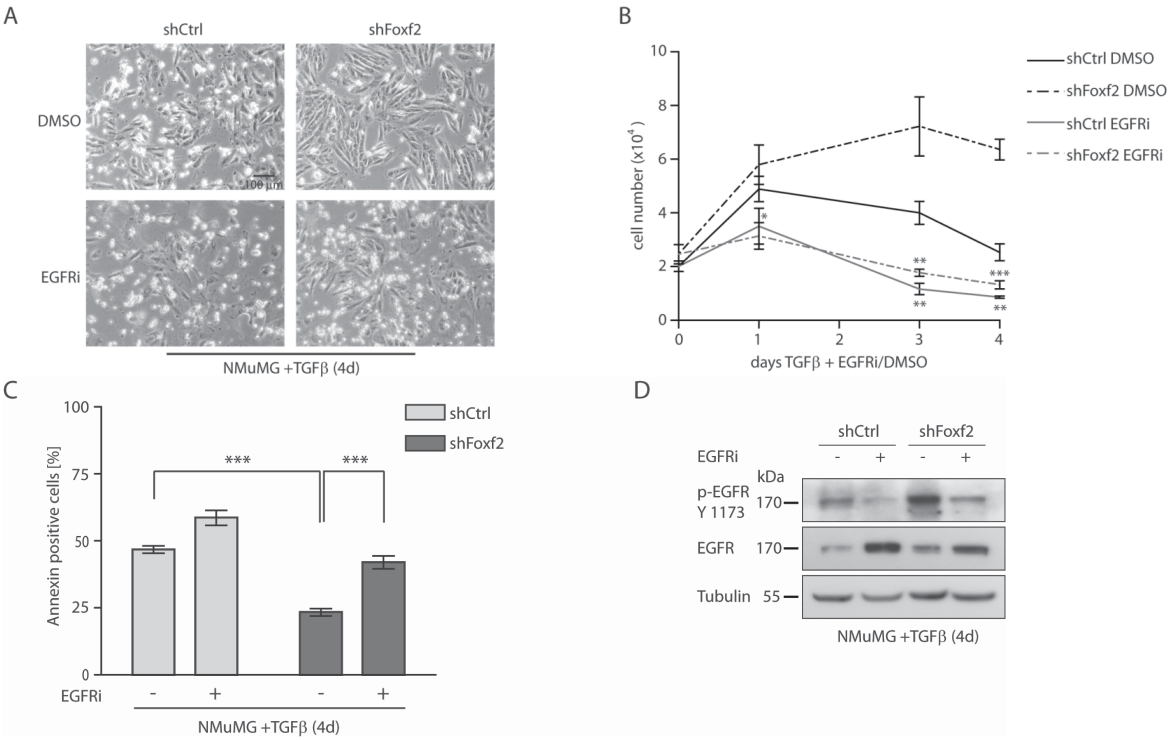


Figure 17: Inhibition of EGFR increases apoptosis in Foxf2 depleted cells. (A-D) NMuMG cells expressing shRNA specific for Foxf2 or control shRNA were treated with TGFβ and AG 1478 (EGFRi) or control solvent (DMSO) for the indicated times. (A) Phase contrast micrographs of NMuMG cells expressing shRNA specific for Foxf2 (shFoxf2) or control shRNA (shCtrl) treated with EGFR inhibitor (EGFRi) or solvent control (DMSO). (B) The inhibition of EGFR activity leads to accelerated cell growth. Cell numbers were determined using a Neubauer chamber. Results presented as mean ±SEM of three independent experiments. Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***) (C) EGFR inhibition significantly increases apoptosis in Foxf2 knockdown cells. Data shown represent the mean ±SEM of 5 independent experiments. Statistical values were calculated using an unpaired two-tailed t-test. P-values ≤ 0.001 indicated with (***). (D) Treatment with AG 1478 decreases EGFR activation in shFoxf2 cells to a similar extend as seen in TGFβ treated NMuMG cells expressing control shRNA. Immunoblotting analysis for EGFR phosphorylation and total EGFR levels. Tubulin was used as a loading control.

4.1.9 Depletion of Betacellulin reduces cell growth in Foxf2 knockdown cells

To assess whether Betacellulin was responsible for the stimulation of EGFR and increased cell survival of the Foxf2 depleted cells, we transfected NMuMG cells stably expressing Foxf2 specific or control shRNA with siRNAs against Betacellulin and treated additionally with TGFβ. The extent of Betacellulin ablation was determined by quantitative RT-PCR (Figure 18B). Reduction of Betacellulin mRNA levels resulted in an ablation of cell growth in shFoxf2 expressing cells when treated with TGFβ (Figure 18C) indicating that the perpetuation of Betacellulin expression in Foxf2-depleted cells is at least partly responsible for the TGFβ-resistant cell growth. To our surprise, the attenuation of Betacellulin resulted in a reduction of apoptosis in both, the shFoxf2 and the control shRNA expressing NMuMG cells (Figure 18D).

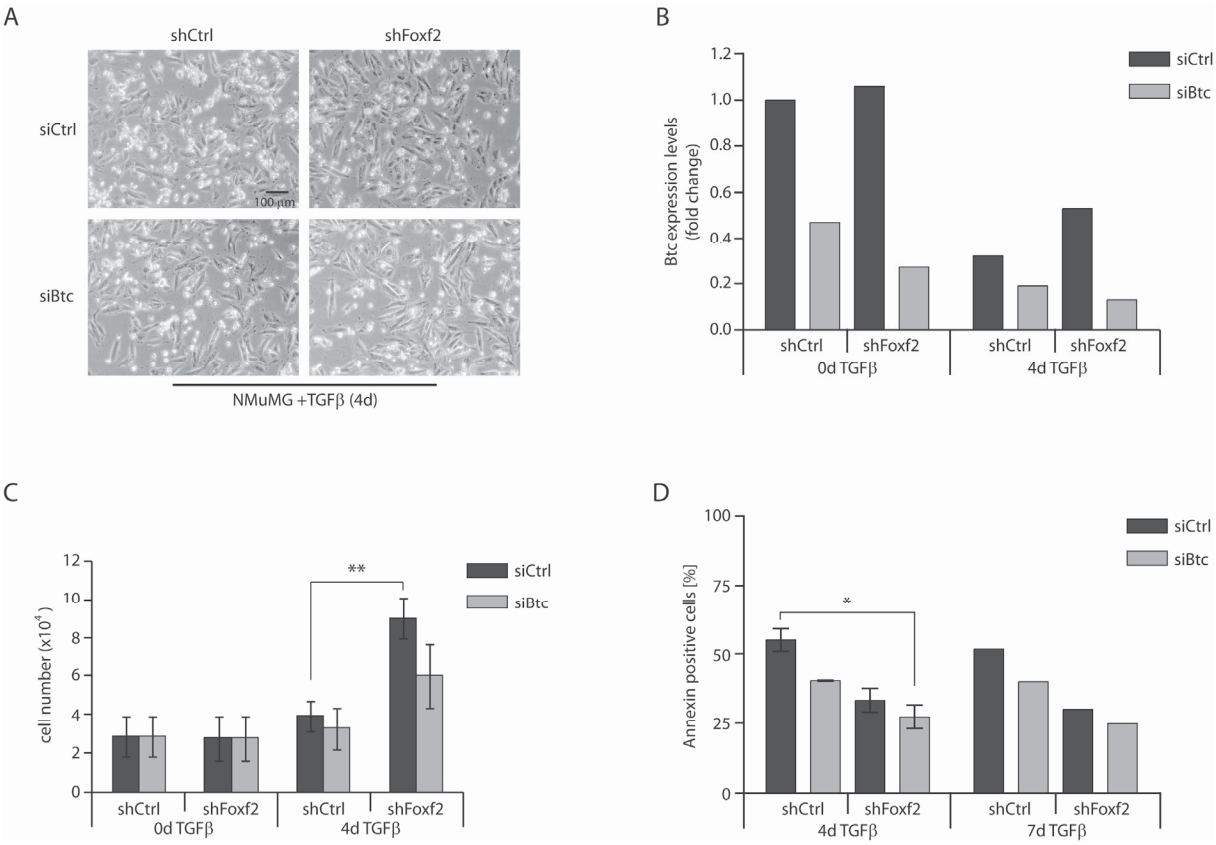


Figure 18: Depletion of the EGFR ligand Betacellulin reduces cell growth. (A-D) Betacellulin expression was downregulated by the use of siRNA in NMuMG cells expressing shRNA specific for Foxf2 or control shRNA. TGFβ treatment was started 24h after siRNA transfection for the time indicated. (A) Phase contrast micrographs of shFoxf2 of shCtrl NMuMG cells transfected with siRNA specific for Betacellulin (siBtc) or control siRNA (siCtrl). (B) Betacellulin mRNA expression levels were determined by quantitative RT-PCR and values were normalized to endogenous RPL19. (C) Betacellulin depletion leads only to a minor reduction in cell growth in Foxf2 knockdown cells treated with TGFβ. Cell numbers were determined using a Neubauer chamber. Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤ 0.01 indicated with (**), (D) Transfection of NMuMG cells with siRNA specific for Betacellulin reduces the levels of apoptosis. Apoptosis was detected by Annexin V staining and FACS analysis. Results are presented as mean ±SD of two independent experiments (4 days TGFβ) or of a single experiment (7 days TGFβ). Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*).

4.1.10 Combined ablation of Betacellulin, Epiregulin and Amphiregulin expression inhibits EGFR mediated survival signaling

To evaluate whether a concomitant downregulation of the three EGFR ligands Betacellulin (Btc), Epiregulin (Ereg) and Amphiregulin (Areg) abrogates the protective effect of the Foxf2 depletion, we transfected stable shFoxf2 and shControl NMuMG cells with siRNAs specific for Betacellulin, Epiregulin and Amphiregulin and induced growth inhibition and apoptosis by treating with TGFβ. The extent of expression repression was determined by quantitative RT-PCR (Figure 19B). Reduction of expression of the three EGFR ligands caused an ablation of cell growth in Foxf2 depleted NMuMG cells treated with TGFβ, mimicking the

growth arrest observed in control NMuMG cells (Figure 19C). Furthermore we detected an increase in apoptosis upon ligand depletion. This result indicated that attenuation of EGFR activation by repression of ligand expression abrogated the survival benefit of Foxf2 downregulation (Figure 19D). In summary, upon TGFβ treatment Foxf2 mediated an impairment of EGFR activation by transcriptional repression of its ligands Betacellulin, Epiregulin and Amphiregulin, leading to an ablation of survival signaling.

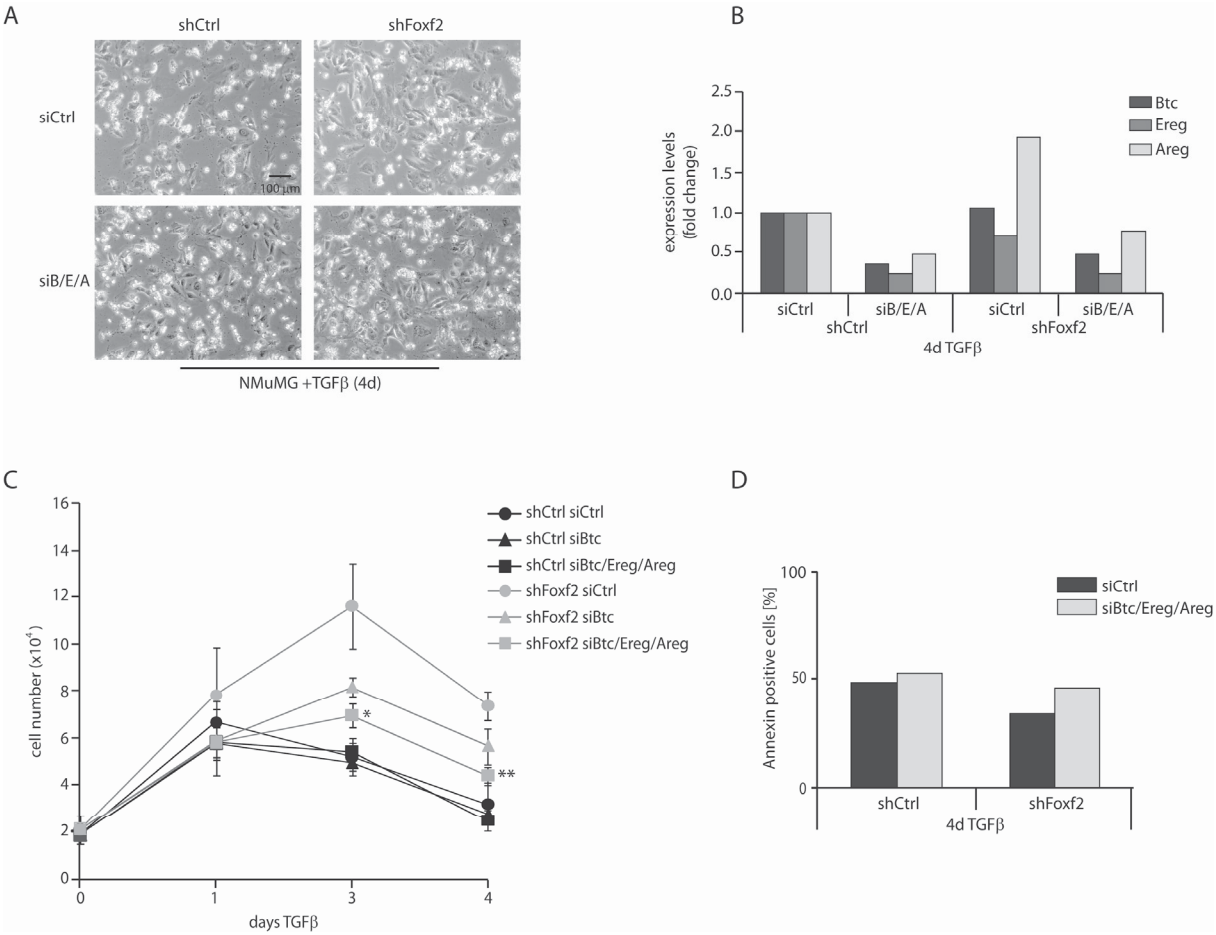


Figure 19: Concomitant knock-down of Betacellulin, Epiregulin and Apmphiregulin decreased cell growth and enhanced TGFβ mediated apoptosis. (A-D) Combined depletion of Betacellulin Epiregulin and Amphiregulin was achieved by the use of siRNA in NMuMG cells expressing Foxf2 specific or control shRNA. TGFβ treatment was started 24h after siRNA transfection for the time indicated. (A) Phase contrast micrographs of shFoxf2 and shCtrl NMuMG cells transfected either with control siRNA (siCtrl) or a combination of Betacellulin, Epiregulin and Amphiregulin siRNA (siB/E/A). (B) mRNA expression levels of the three EGFR ligands Betacellulin (Btc), Epiregulin (Ereg) and Amphiregulin (Areg) were determined by quantitative RT-PCR, normalized to endogenous RPL19. (C) Contiguous depletion of Btc, Ereg and Areg leads to reduced cell growth in stable Foxf2 knockdown cells. Cell numbers were determined using a Neubauer chamber. Statistical values were calculated in relation to shFoxf2 siCtrl, by using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**) (D) Downregulation of the three EGFR ligands leads to an increase in TGFβ induced apoptosis. Apoptosis was detected by Annexin V staining and FACS analysis.

4.1.11 High expression levels of Foxf2 correlates with poor prognosis for ER-negative tumors

Cancer-associated gene-expression profiling has emerged to be an appropriate tool to predict the relapse risk and to identify genes that mediate disease progression. To investigate whether Foxf2 is predictive for tumor progression or metastasis formation, we analyzed the Netherlands Cancer Institute (NKI295) breast cancer database for Foxf2 expression (269). The NKI295 database consists of Microarray gene expression analysis of tumor samples from 295 patients with early stage breast cancer (stage I or stage II primary breast carcinomas, determined by H&E staining). The expression levels of the investigated genes are listed as fold change induction normalized to the expression of the pooled tumors. Although Foxf2 expression was not predictive for metastasis formation or survival in the total patient pool, high expression of Foxf2 in tumors with negative estrogen receptor (ER) status correlated with high significance with early metastasis onset as well as poor overall survival (Figure 20B,C). This outcome indicates that high expression of Foxf2 in ER-negative mammary carcinomas correlates with poor prognosis for tumor progression and patient survival.

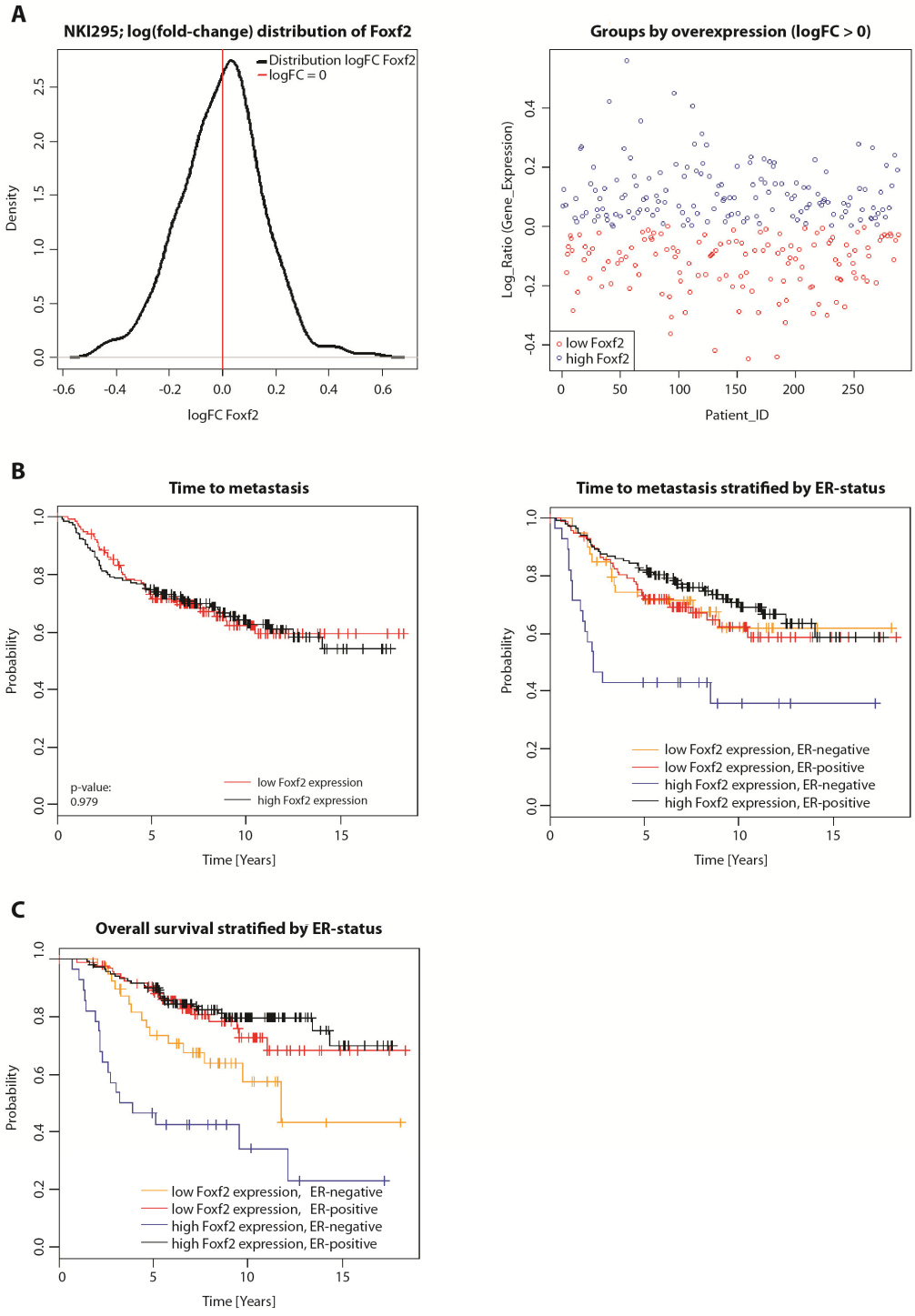


Figure 20: High Foxf2 expression correlates with faster metastases formation and worse overall survival in ER-negative tumors. Statistical analysis of the Netherlands Cancer Institute (NKI295) database was performed by Nathalie Meyer-Schaller. (A) Tumors were divided into high and low Foxf2 expressing groups based on the relative expression of Foxf2 to the tumor pools (logFC = 0). (B/C) Expression of Foxf2 was correlated with time to metastasis (B) or overall survival (C) either in all tumors analyzed (N=288) or specifically in ER-negative tumors (N=68) and ER-positive tumors (N=220). Foxf2 expression does not correlate with tumor metastasis of the total tumor pool but Foxf2 expression is predictive for metastasis incidence in ER-negative tumors were high expression of Foxf2 correlates with an early onset of metastases (p-value = 6.53e-03). Consistently high Foxf2 expression levels correlate with shorter overall survival in ER-negative tumors (p-value = 0.0154).

4.1.12 Correlation of Foxf2 expression with metastasis free survival depends on LN status

To further substantiate a potential correlation between Foxf2 expression and patient survival, we analyzed a breast cancer database of the Memorial Sloan-Kettering Cancer Center (MSKCC), published by Minn et al. (57). This "Minn" database consists of microarray gene expression analysis of tumor samples from 82 patients with more advanced breast cancer (T2-T4). The tumors were divided into two groups according to the log expression levels relative to the median expression of the investigated genes (Figure 21A). The low and high expressing groups were further stratified for lymph node (LN) status. Interestingly, low Foxf2 expression significantly correlated with early distant metastasis formation in LN^- tumors, whereas the opposite tendency was found in tumors of patients, that were positive for lymph node metastases (Figure 21B). This tendency is even more obvious, although not significant (probably due to low sample number), when the correlation of Foxf2 expression to bone marrow metastases formation was analyzed (Figure 21C). This outcome reflects the dual function of Foxf2, seen in the *in vitro* studies, - while Foxf2 might function as tumor suppressor in early cancer development by promoting apoptosis , hence showing a bad prognosis in LN^- patients with low Foxf2 expression - more advanced tumors with high Foxf2 expression correlates with shorter metastasis free survival, further substantiating a role of Foxf2 in cancer cell invasion and metastases formation.

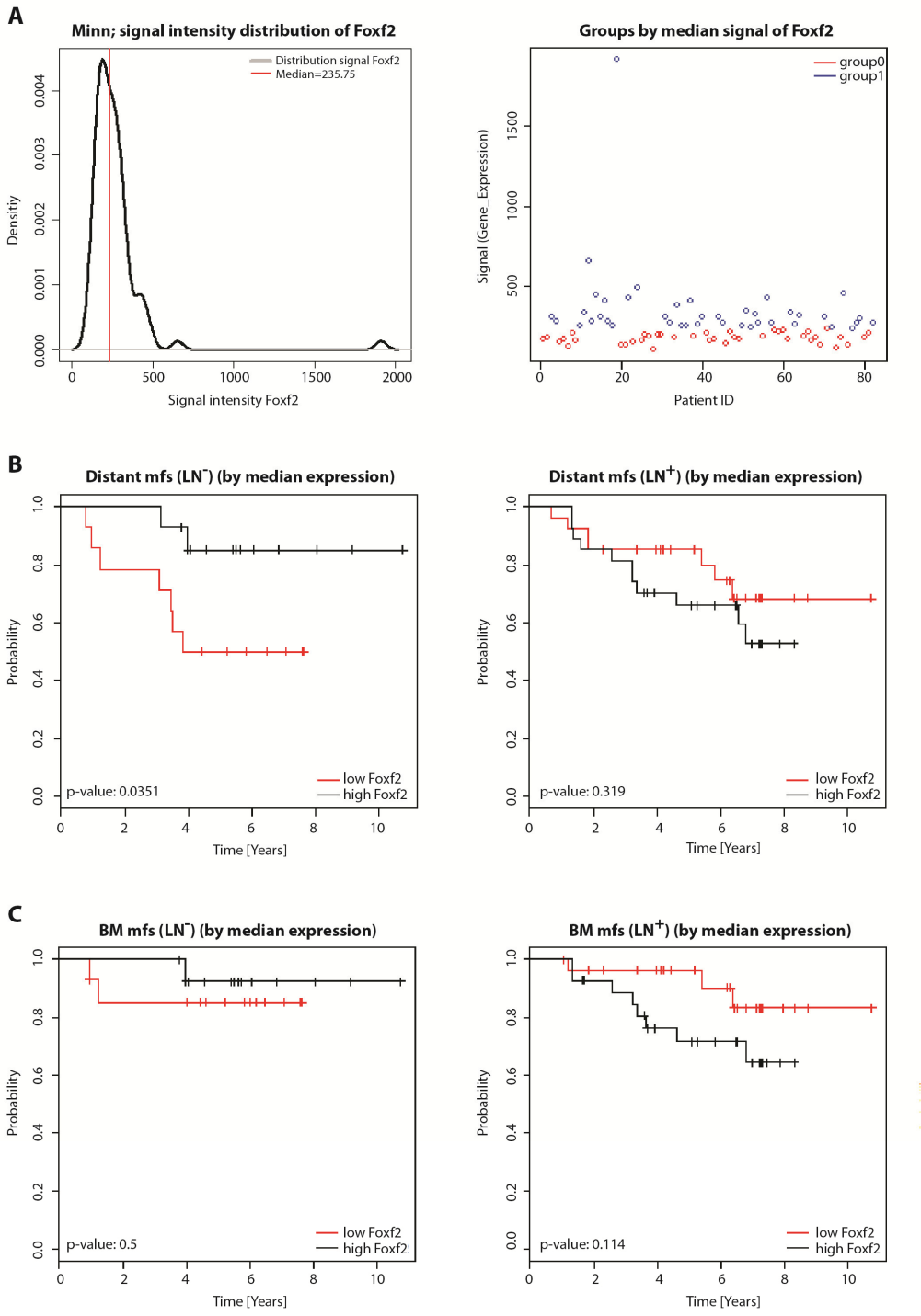


Figure 21: Low Foxf2 expression correlates with faster metastases formation in LN-negative tumors, whereas high Foxf2 expression correlates in tendency with bad prognosis in LN-positive tumors. Statistical analysis of the Memorial Sloan-Kettering Cancer Center (Minn) database was performed by Nathalie Meyer-Schaller. (A) Tumors were divided into high and low Foxf2 expressing groups based on the median expresson of Foxf2. (B) Expression of Foxf2 was correlated with distant metastasis free survival (mfs) in LN^- tumors (N=28) or LN^+ tumors (N=54). Foxf2 expression is predictive for metastasis incidence in LN-negative tumors, where low Foxf2 expression correlates with early metastases onset (p-value = 0.0351). (C) Correlation of Foxf2 expression with bone marrow (BM) metastasis free survival (mfs) was investigated stratified by LN status. High Foxf2 expression correlates in tendency with BM metastasis, where high Foxf2 levels are indicative for earlier metastasis (p-value = 0.114).

4.2 Discussion

Overcoming the growth inhibitory effect of TGFβ during tumor onset and early stages of tumor development as well as the ability of advanced tumors to convert TGFβ responsiveness in cancer cells to gain invasive properties are important incidences during tumor growth and metastasis formation (65, 84). Thus, understanding the mechanisms behind this dual role of TGFβ in cancer progression and the strategies of cancer cells to circumvent TGFβ-induced apoptosis offers potential for new cancer therapies. *In vitro* studies of the molecular mechanisms of the EMT process in non-transformed NMuMG cells facilitate the dissection of the individual processes during EMT, including resistance to apoptosis and acquisition of invasive properties.

In the present study, we generated an EMT gene signature by use of different *in vitro* EMT model systems to identify the transcription factor Foxf2 as a commonly upregulated gene in cells undergoing EMT. By studying TGFβ-treated NMuMG cells as a flexible EMT-model system and human breast cancer gene expression profiling databases, we depicted a dual function of Foxf2, acting as a tumor suppressor by promoting apoptosis as well as a pro-migratory function by inducing the disruption of cell-cell adhesion.

Foxf2 is upregulated via the canonical TGFβ pathway and gain of function studies in NMuMG cells revealed that its expression is not sufficient to induce EMT. Interestingly, loss of function studies showed that Foxf2 is not required for the gain of mesenchymal markers but its upregulation is essential for the disruption of cell junctions and downregulation of epithelial markers. Furthermore, we found that Foxf2 function is crucial for TGFβ-mediated cell death via upregulation of BH3-only-mediated caspase-dependent apoptosis and attenuation of EGFR-mediated survival signaling.

The failure of Foxf2-depleted cells to disrupt cell-cell contacts by downregulation of tight- and adherens junction components displays its indispensable role in the acquisition of an invasive single cell morphology. Loss of E-cadherin is an early event during EMT resulting in the disruption of the polarity complex, a prerequisite for the dissociation and invasion of cancer cells (98, 121). Direct and indirect transcriptional repression via mediators, such as ZEB1, ZEB2 and Id2, have emerged as common regulatory mechanisms of E-cadherin

expression in various cancer types (108, 113). By mediating the TGFβ-induced upregulation of the transcriptional repressors ZEB1 and ZEB2 as well as the repression of Id2, Foxf2 causes the transcriptional downregulation of E-cadherin and consequently the disruption of cell-cell adhesion. The mechanism by which Foxf2 regulates the expression of these transcription factors remains elusive. Interestingly, recent findings identified the miR-200 family as potent EMT-inducing non-coding RNAs targeting ZEB1 and ZEB2 transcripts (93, 94). The potential role of further non-coding RNAs during EMT is under current investigation in our laboratory. The failure of TGFβ to induce adherens junction breakdown in Foxf2-depleted cells results in the preservation of tight cell-cell contacts and, consequently, in a clear reduction in cell motility, suggesting a role for Foxf2 as a pro-metastatic factor.

Furthermore, our results show that Foxf2 upregulation is essential for TGFβ-mediated apoptosis. The reduction of TGFβ-induced apoptosis in Foxf2 deficient cells is a consequence of the loss of the direct transcriptional activation of Noxa expression. The pro-apoptotic protein is highly upregulated by TGFβ, activating classical mitochondrial caspase-dependent cell-death, and we have been able to show that its upregulation is indispensable for apoptosis induction in NMuMG cells. The BH-3 only protein Noxa has been shown to be crucial in fine-tuning cell death decisions via degradation of the pro-survival molecule Mcl1 (281). Although Noxa has been identified as a primary p53-response gene, oncogenic stress, such as irradiation and hypoxia, resulted in efficient induction of Noxa also in the absence of p53 (282, 283). Thus, our findings identify Foxf2 as a novel transcriptional activator of the tumor suppressor Noxa.

Besides triggering apoptosis via the regulation of Noxa expression, Foxf2 is also involved in the negative regulation of survival signals by downregulation of EGFR protein levels as well as transcriptional repression of its ligands Betacellulin and Amphiregulin. This leads to a reduction of EGFR phosphorylation and hence, reduced PKB activation. Blocking of EGFR signaling has been shown to amplify the apoptotic response to TGFβ (192). Here, we show that both, receptor inhibition by use of a chemical inhibitor as well as repression of the three EGFR ligands Betacellulin, Epiregulin and Amphiregulin increased TGFβ-induced apoptosis in Foxf2 depleted NMuMG cells. These results indicate that, in addition to Noxa regulation, Foxf2 mediates its apoptotic effect by blocking EGFR-mediated survival signaling.

Whether the TGFβ-mediated and Foxf2-dependent reduction in survival signaling is due to reduced ligand expression and/or depends on increased EGFR internalization and degradation needs further investigation.

To support the importance of a Foxf2 function during tumor development and progression, we have performed correlation studies for Foxf2 on two breast cancer databases (57, 269). Analysis of the lymph node negative stratified tumor subset demonstrated that low Foxf2 expression significantly correlates with early metastasis formation. Interestingly, the opposite tendency is found in more malignant tumors displaying lymph node metastases, where high Foxf2 expression correlates with worse prognosis. Moreover, studies on tumors, stratified for the ER status reveal a highly significant correlation of high Foxf2 expression and early metastasis onset, coherent with worse overall survival. The ER^- tumors represent a more aggressive breast cancer subtype, thus identifying high levels of Foxf2 as a marker correlating with good prognosis in early non-invasive stages of tumor development, but with poor prognosis in a highly malignant background (22). These findings substantiate the dual role of Foxf2 and its implication in metastasis formation.

Stable shRNA-mediated downregulation of Foxf2 showed an attenuation but not a complete abrogation of the observed TGFβ-mediated phenotypes. As in all presented results the function of Foxf2 was clearly dose dependent, a knockout of the transcription factor would probably be more conclusive about its role in EMT and cancer progression. However under pathological conditions, complete depletion of a gene is not always physiological and therefore less realistic to investigate. In this study we show that Foxf2 is a transcription factor with a dual function: on one hand it can act, most likely with the help of its cofactors, as a tumor suppressor by inducing apoptosis. On the other hand, as a pro-metastatic factor by conferring migratory capabilities to the cells which have overcome the apoptotic crisis and undergone EMT. Foxf2's role in pre- and post-EMT cells reflects the well-studied dual role of TGFβ in cancer progression. Our results also substantiate findings in knockout mouse models where Foxf2 was found to play an important role in EMT-associated developmental processes and maintenance of the epithelial-mesenchymal structure in lung and gut tissues (257, 258). Hence, fine-tuning of the expression of Foxf2 and its cofactors could be pivotal

during cancer development and, thus, better insights in its regulation and molecular function is potentially beneficial for future therapeutical strategies.

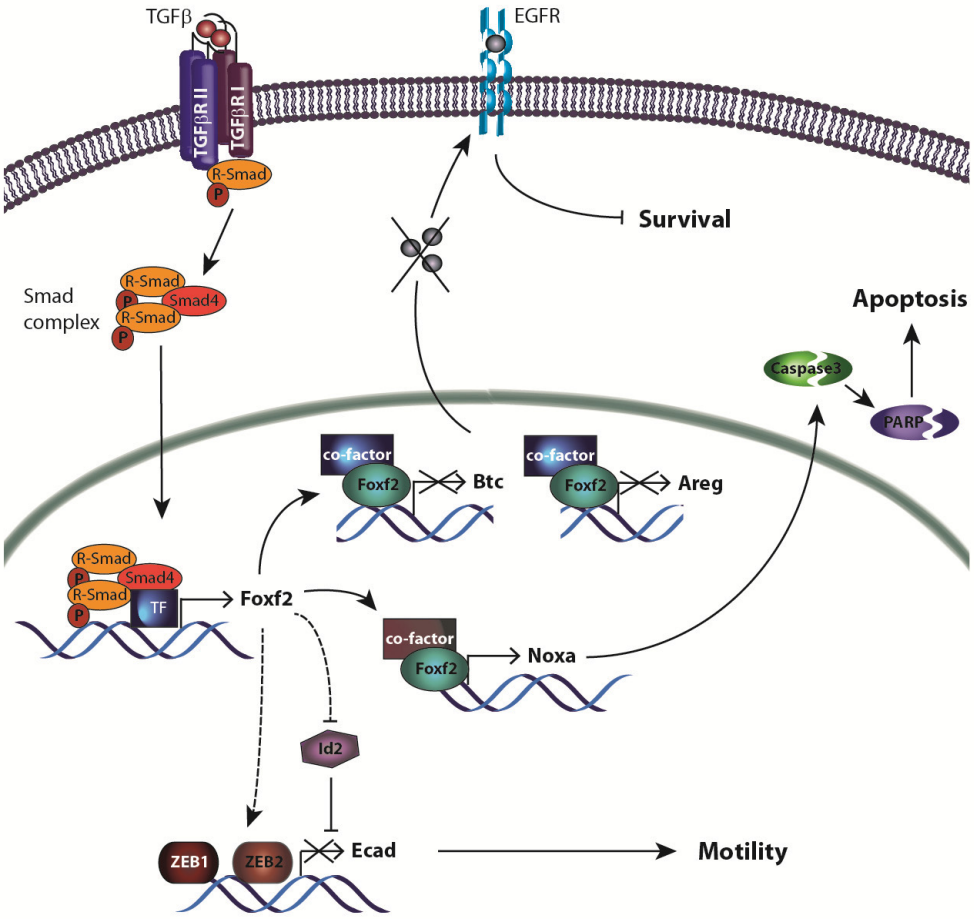


Figure 22: Schematic model of TGFβ-mediated Foxf2 upregulation reflecting its dual role during EMT. Receptor activation by TGFβ leads to phosphorylation of receptor-bound Smads with subsequent Smad complex formation. The trimeric complex enters the nucleus to activate the expression of TGFβ target genes, such as the forkhead transcription factor Foxf2. Subsequently, Foxf2 directly represses the transcription of the EGFR ligands Betacellulin (Btc) and Amphiregulin (Areg), which leads to reduced EGFR-mediated survival signaling. Furthermore, Foxf2 directly binds and transcriptionally activates the pro-apoptotic gene *NOXA*, resulting in the induction of Caspase-dependent apoptosis. On the other hand, Foxf2 leads to the downregulation of E-cadherin (Ecad) by upregulating the transcriptional repressors ZEB1 and ZEB2 and downregulating the Ecad activator Id2, leading to the disruption of adherens junctions and subsequent increase of migratory capabilities.

5 N-cadherin - a marker without function?

5.1 Results

5.1.1 Ablation of N-cadherin expression does not prevent TGFβ-induced EMT in NMuMG cells

A hallmark of EMT is the change of expression from epithelial E-cadherin to the expression of mesenchymal N-cadherin, which is accompanied with alterations in adhesive properties, as cells lose their affinity for epithelial neighbors and gain affinity for stromal cells (35). The cadherin switch induces cell migration and invasion and is suspected to support the transition from benign to invasive malignant tumors (118-120). Loss of E-cadherin function has been shown to be required and sufficient to induce EMT and increase motility and invasiveness in several cell systems (262, 284, 285). As the loss of E-cadherin is an inducer of EMT and the cadherin switch a widely observed phenomenon during this transition, we wanted to assess whether the expression of N-cadherin is of comparable importance in this process. To investigate, whether N-cadherin (Ncad) depletion leads to a loss of mesenchymal phenotype or even the induction of MET, mesenchymal cells were transiently transfected with siRNA specific for N-cadherin. Ablation of N-cadherin in mesenchymal murine MTΔEcad cells did not lead to alterations in the spindle-like fibroblastoid phenotype, even though a substantial knock down efficiency was achieved (Figure 23A/B). Due to the stable and complete ablation of E-cadherin by Cre-recombinase, these cells can be regarded as an endpoint EMT system lacking the flexibility to undergo MET. To use a more transient model, we depleted N-cadherin in mesenchymal NMuMG cells intitially treated for more than 30 days with TGFβ (cells known as NMuMG LT) to assess potential phenotypical changes. However, N-cadherin downregulation did neither result in alterations of the cell shape nor in an increase of E-cadherin expression, which would both indicate an induction of MET (Figure 23C/D).

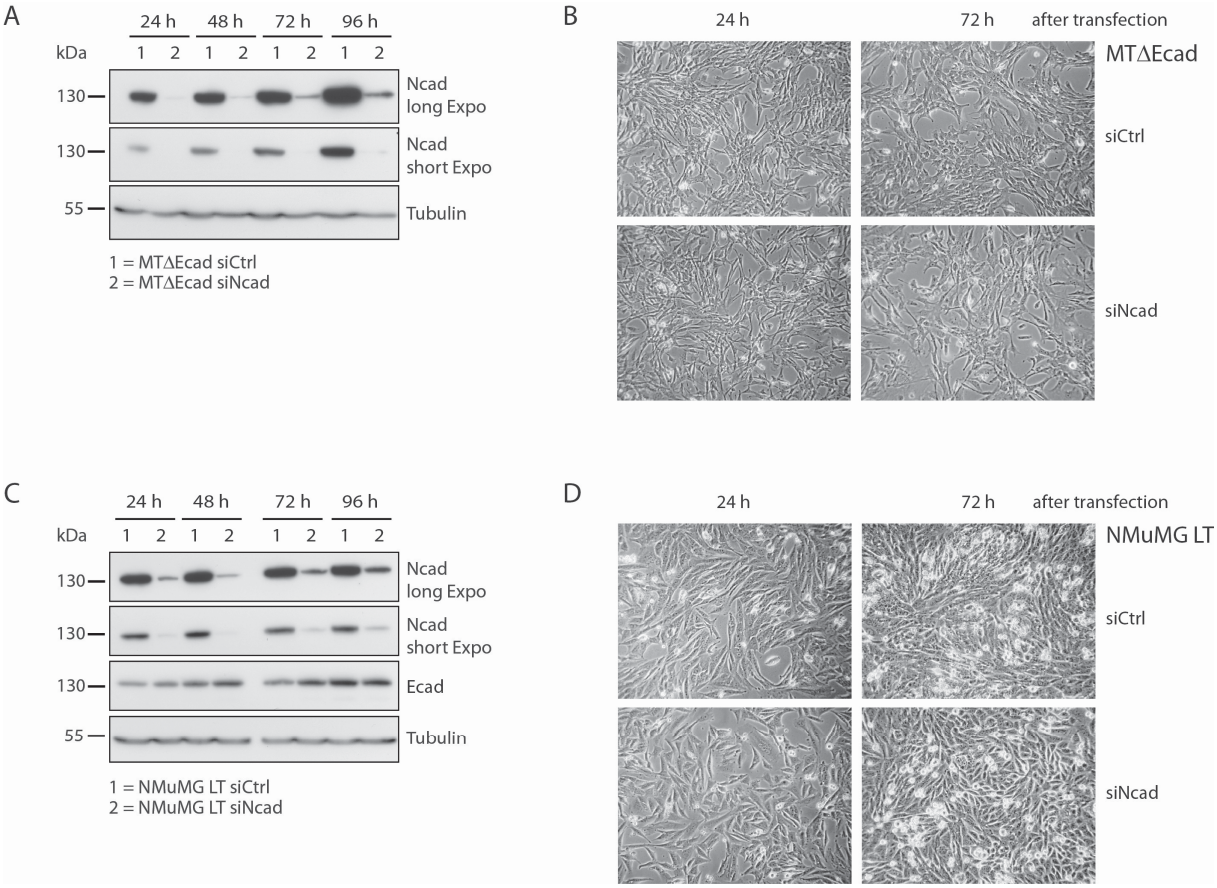


Figure 23: N-cadherin ablation does not induce MET. (A/B) N-cadherin expression was depleted in mesenchymal MTΔEcad by use of siRNA. (A) Knockdown efficiency at indicated timepoints after transfection with either control (siCtrl) or N-cadherin specific (siNcad) siRNA was analyzed by immunoblotting. Tubulin was used as a loading control. (B) MTΔEcad cells at indicated time after siRNA transfection were analyzed by phase contrast microscopy. (C/D) N-cadherin expression was downregulated in mesenchymal NMuMG LT cells by use of siRNA. (C) Knockdown efficiency as well as the expression of the epithelial marker E-cadherin at indicated timepoints after transfection was determined by immunoblotting. Tubulin was used as a loading control. (D) Phase contrast images of NMuMG LT cells at indicated time after siRNA transfection.

To assess whether the ablation of N-cadherin expression is sufficient to prevent EMT, NMuMG cells were transfected with siRNA specific for N-cadherin. NMuMG-siNcad cells treated with TGFβ show a clear change in morphology by gaining an elongated fibroblastoid phenotype as seen in the control siRNA (siCtrl) (Figure 24A). To investigate whether the depletion of N-cadherin had an effect on the loss of epithelial markers and the gain of mesenchymal markers, we analyzed the expression of adherens junction proteins E-cadherin, p120-catenin and β-catenin and the mesenchymal marker NCAM. Immunoblotting analysis as well as quantitative RT-PCR revealed that suppression of N-cadherin upregulation in TGFβ-induced EMT had neither an impact on the downregulation of epithelial markers nor on the upregulation of mesenchymal proteins (Figure 24B/C).

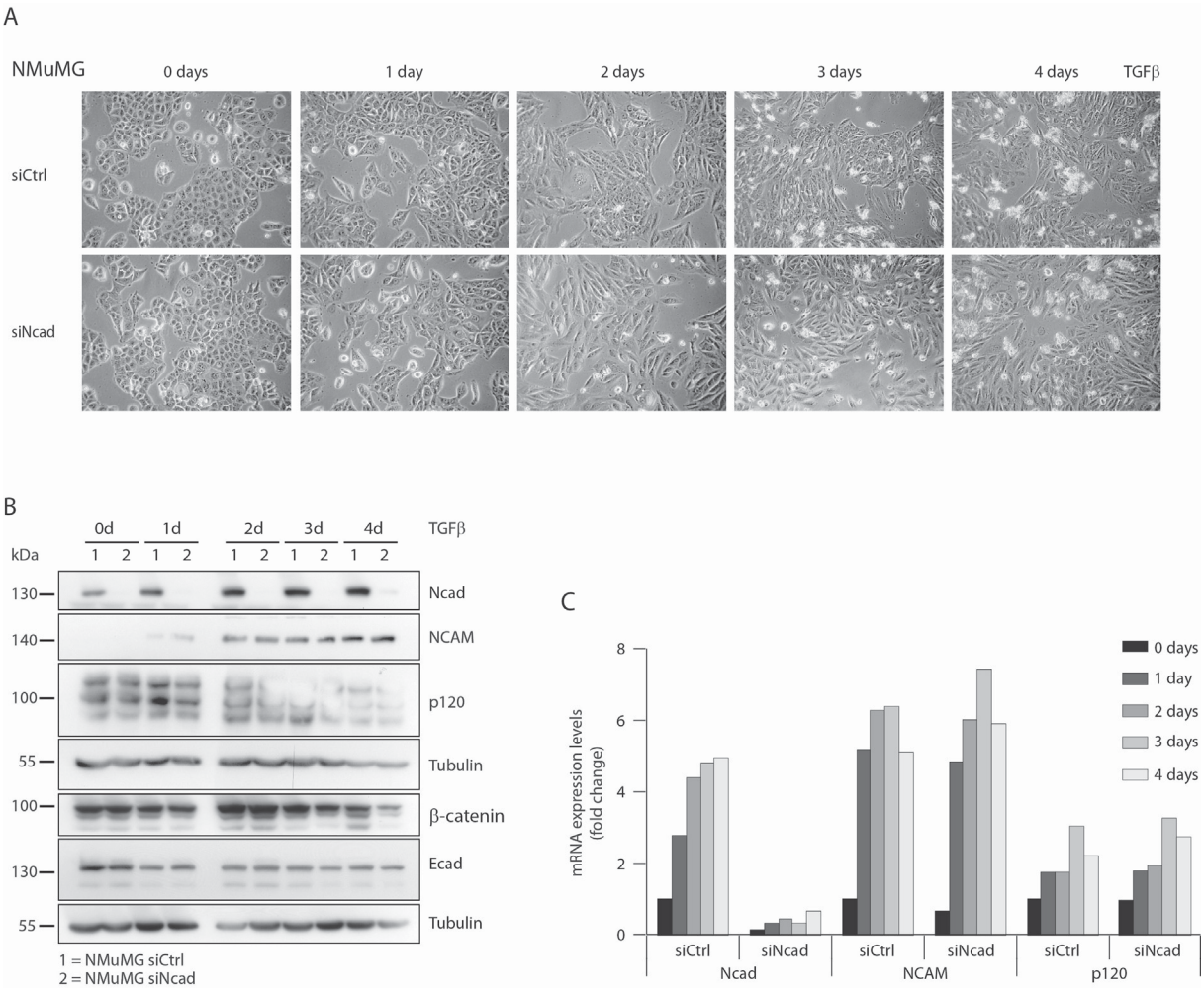


Figure 24: Ablation of N-cadherin is not sufficient to prevent TGFβ-induced EMT. (A) Phase contrast images of NMuMG cells transfected with control siRNA (siCtrl) or N-cadherin specific siRNA (siNcad), and treated with TGFβ for the indicated times. (B) Immunoblot analysis of the epithelial markers E-cadherin (Ecad), p120-catenin (p120) and β-catenin as well as the mesenchymal markers N-cadherin (Ncad) and NCAM in NMuMG cells transfected with siCtrl or siNcad, treated with TGFβ for the times indicated. Tubulin was used as a loading control. (C) Expression levels of N-cadherin, NCAM and p120-catenin were determined by quantitative RT-PCR. Values were normalized to endogenous murine RPL19.

5.1.2 Downregulation of N-cadherin does not affect the cytoskeletal composition

To investigate whether a downregulation of N-cadherin during EMT has an impact on EMT-associated alterations in cell adhesion and/or cytoskeletal composition, we performed an immunofluorescence staining for the cell adhesion proteins E-cadherin, N-cadherin, p120-catenin and β-catenin, and the actin cytoskeleton (phalloidin). In NMuMG cells treated with TGFβ and transfected with control siRNA (siCtrl), a normal upregulation of the mesenchymal marker N-cadherin was observed, which was repressed efficiently by the transfection with siRNA specific for N-cadherin (siNcad) (Figure 25). In the N-cadherin depleted cells, we

observed a disorganized localization of the adherens junction protein β-catenin, which is normally functioning as a linker protein between cadherin proteins and the actin cytoskeleton. The disorganization is probably due to the lack of binding partners as N-cadherin is depleted and E-cadherin is internalized and transcriptionally repressed during the EMT process.

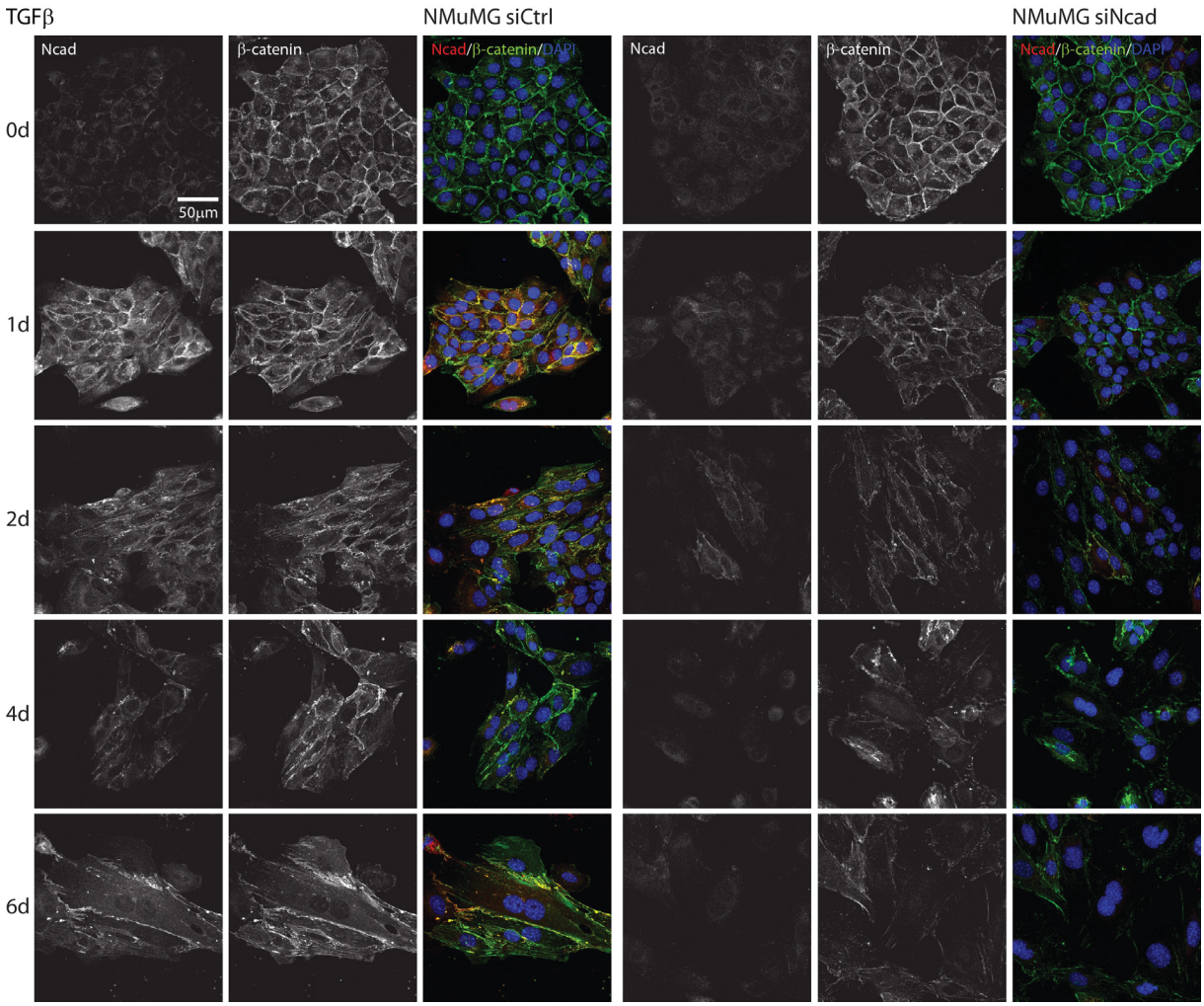


Figure 25: Downregulation of N-cadherin results in reduced β-catenin localization at the cell membrane. Maximum intensity projection of confocal stacks of NMuMG cells transfected with control siRNA (siCtrl) or siRNA specific for N-cadherin (siNcad) treated with TGFβ for the indicated times, stained for N-cadherin (red) and β-catenin (green). The nuclei are visualized by staining with DAPI (blue).

Indeed, we were able to show that the downregulation of N-cadherin does not influence the loss of E-cadherin during TGFβ-induced EMT (Figure 26). Concomitant with the downregulation of E-cadherin we observed a relocalization of p120-catenin from the cell membrane to the cytoplasm in NMuMG cells independent of the expression of N-cadherin.

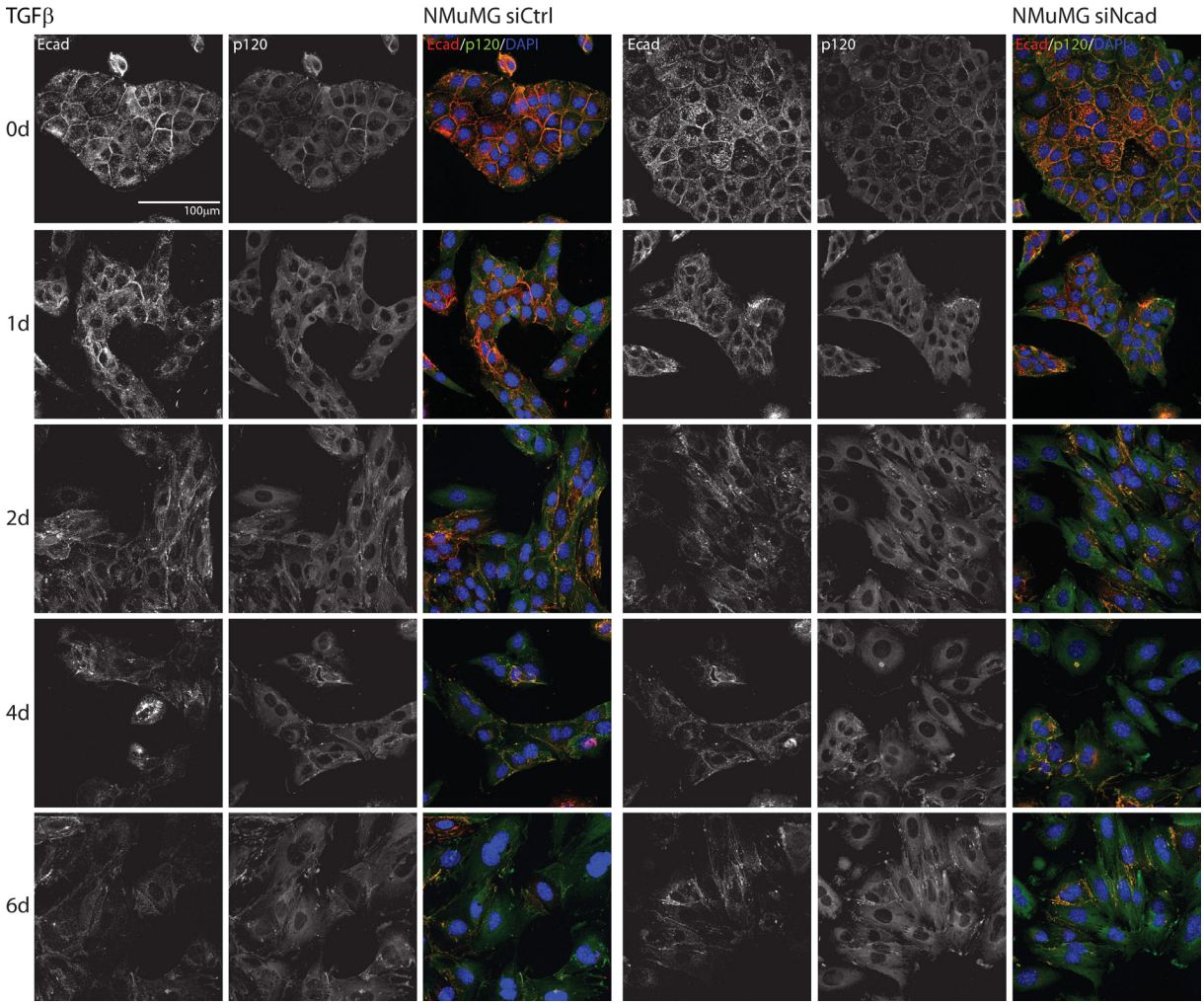


Figure 26: Depletion of N-cadherin does not affect E-cadherin downregulation and p120-catenin localization during EMT. Maximum intensity projection of confocal stacks of NMuMG cells transfected with control siRNA (siCtrl) or siRNA specific for N-cadherin (siNcad) treated with TGFβ for the indicated times, stained for E-cadherin (red) and p120-catenin (green). The nuclei are visualized by staining with DAPI (blue).

Furthermore, we show that depletion of N-cadherin does not influence the EMT-associated cytoskeletal reorganization. Compared to the control cells, N-cadherin knockdown cells showed a similar rearrangement of the cortical actin to stress fibers resulting in a spindle-like elongated cell shape upon treatment with TGFβ (Figure 27). In summary, the data show that the upregulation of N-cadherin during EMT is not essential and that the depletion of N-cadherin in mesenchymal cells is not sufficient to induce MET.

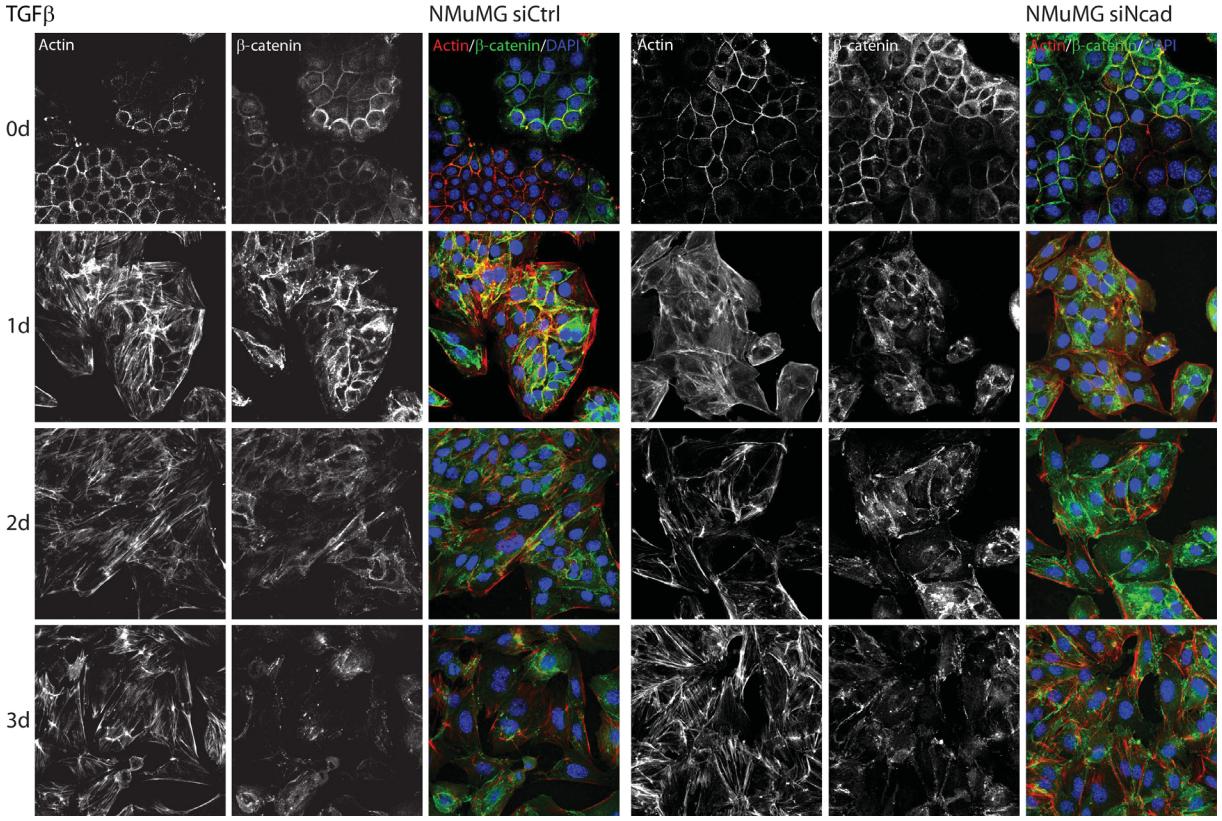


Figure 27: N-cadherin knockdown does not affect stress fiber fomation during TGFβ-induced EMT. Maximum intensity projection of confocal stacks of NMuMG cells transfected with control siRNA (siCtrl) or siRNA specific for N-cadherin (siNcad) treated with TGFβ for the indicated times, stained for filamentous actin (red) and β-catenin (green). The nuclei are visualized by staining with DAPI (blue).

5.1.3 N-cadherin depletion in mesenchymal cells has no impact on cell motility

The switch from epithelial to mesenchymal cadherin expression is accompanied by alterations of adhesive properties and is suspected to increase the migratory capability of tumor cells (118, 119). We have shown that the downregulation of E-cadherin is required for an EMT-associated increase of motility in NMuMG cells (Figure 13). Hence, we wanted to investigate the importance of N-cadherin upregulation in TGFβ-induced cell migration. We performed a migration assay comparing the migratory capability of N-cadherin knockdown cells to the mesenchymal control cells, using the long term (LT) NMuMG and the MTΔEcad cells. We found in neither cell type an impact of N-cadherin depletion on cell motility.

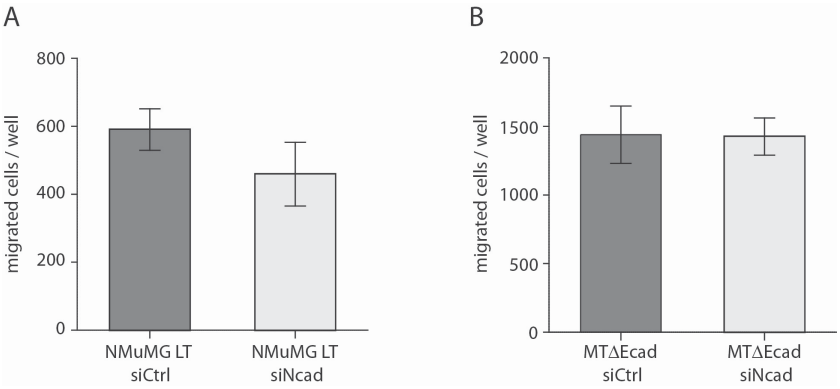
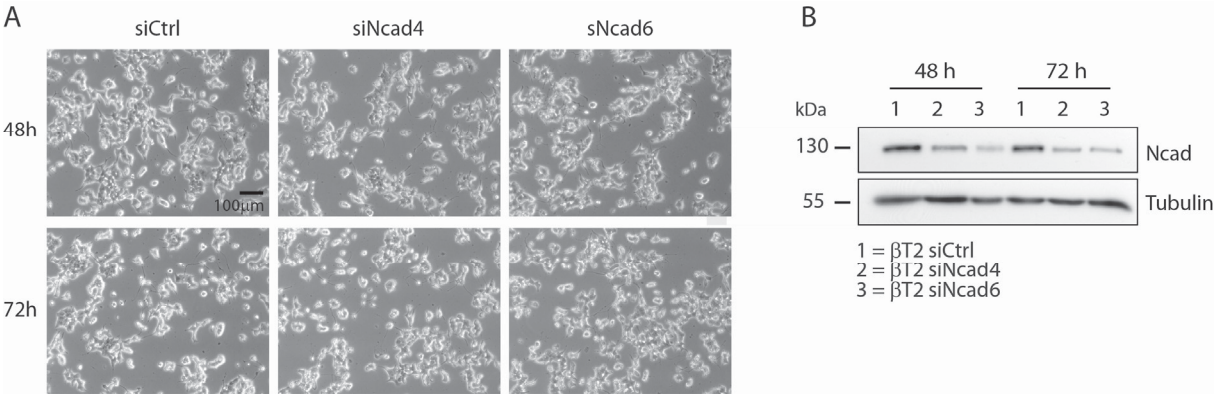


Figure 28: Depletion of N-cadherin has no impact on cell motility of mesenchymal cells. (A) Migration assay with control or N-cadherin-depleted mesenchymal long term (LT) TGFβ-treated NMuMG cells through transwell filters. (B) Migration assay with control or N-cadherin depleted MTΔEcad cells through transwell filters.

5.1.4 N-cadherin depletion does not affect neurite formation in βT2 pancreatic cells

It has been previously shown that the expression of the immunoglobulin family member NCAM is upregulated concomitantly with the loss of E-cadherin function in various cellular systems, and that ablation of NCAM expression inhibits EMT (262). Furthermore, NCAM has been shown to be essential for the formation of cellular protrusions (neurite outgrowth) and the induction of matrix adhesion of β-cells from pancreatic tumors. Both, neurite formation as well as cell-matrix adhesion are dependent on NCAM-induced assembly of a signaling complex consisting of N-cadherin, fibroblast-growth-factor receptor-4 (FGFR-4), phospolipase Cγ (PLCγ), the adaptor protein FRS2, $pp60^{c\text{-}src}$, cortactin and GAP-43 (133). Since NCAM is potentially implicated in the prevention of the dissemination of metastatic tumor cells and its function is essential for the formation of cellular protrusions, we wanted to assess whether the complex component N-cadherin is also required for neurite formation. Using an established cell line derived from Rip1Tag2 induced pancreatic β-cell tumors (βT2 cells), we investigated the effect of N-cadherin depletion on the ability to form cellular protrusions. Although a clear attenuation of N-cadherin protein levels was achieved by the use of two different siRNAs, we did not observe any changes in cell phenotype or neurite formation capacity (Figure 29A/B). Surprisingly, immunofluorescence microscopy analyses not only showed the same frequency of neurite outgrowth in the control and the N-cadherin-depleted βT2 cells but also a preferential neurite formation in the control cells

expressing low level of N-cadherin (Figure 29C). These results suggest that N-cadherin expression is not required for the formation of cellular protrusions in βT2 cells.



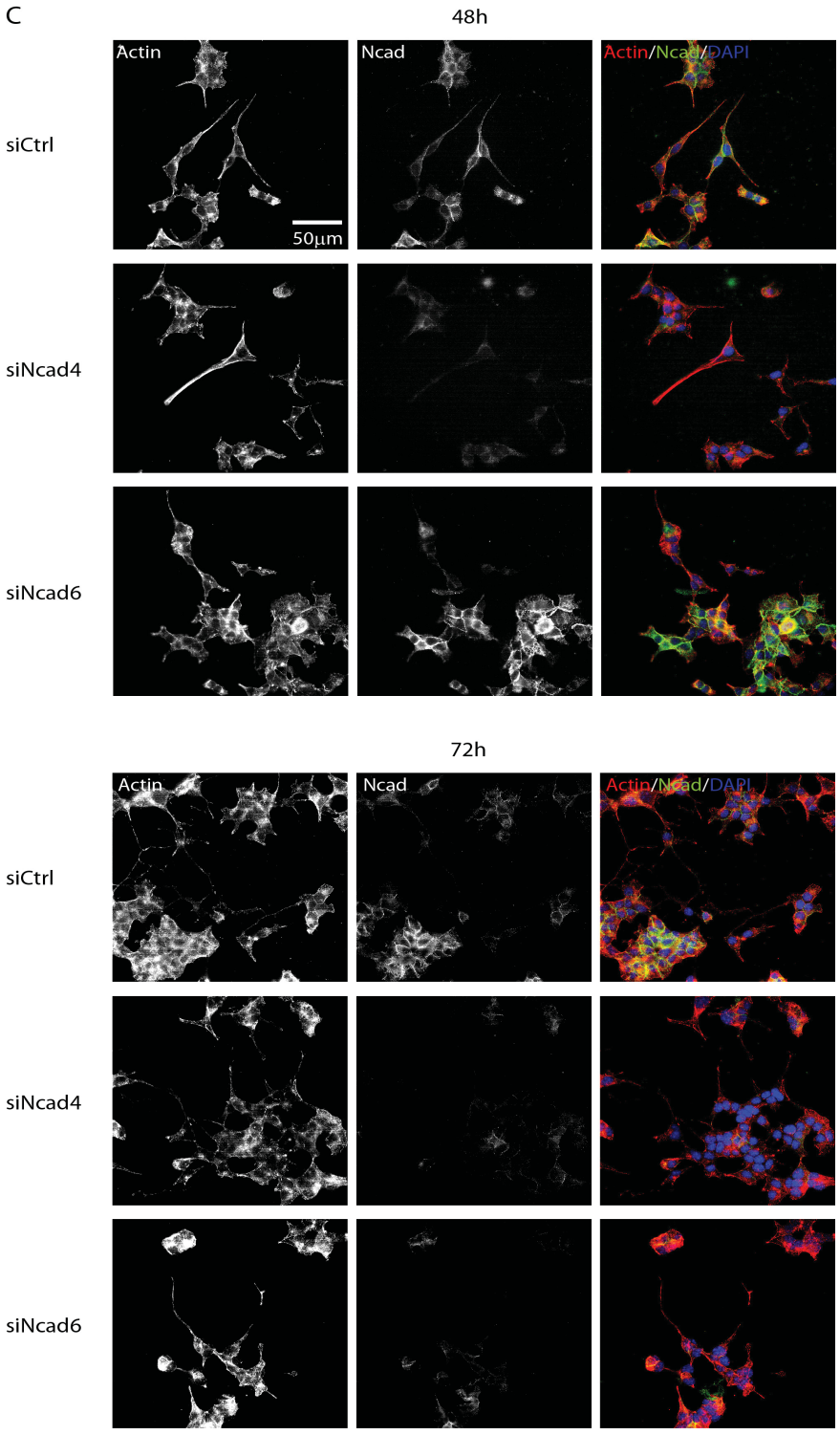


Figure 29: N-cadherin knockdown in βT2 cells does not influence neurite formation capacity. (A) Phase contrast images of β- tumor cells (βT2) transfected with control siRNA (siCtrl) or siRNA specific for N-cadherin (siNcad4, siNcad6). (B) Knock down efficiency was determined by immunoblotting analysis, showing comparable efficiencies for both N-cadherin specific siRNAs. (C) Immunofluorescence microscopy analysis of filamentous actin (red) and N-cadherin (green) of control tumor β-cells (βT2) or βT2 cells with downregulated N-cadherin (siNcad4, siNcad6) at 48 hours and at 72 hours after transfection. The nuclei are visualized by staining with DAPI (blue).

5.2 Discussion

The attenuation of the expression and/or protein function of the cell-cell adhesion molecule E-cadherin is a hallmark of EMT and is found in the majority of epithelial cancers (132). In many cancer types, the loss of E-cadherin is accompanied by a gain of expression of the mesenchymal cadherin N-cadherin. The cadherin switch is thought to be required for tumor cells to acquire invasive properties and N-cadherin is upregulated in aggressive breast tumors, but its function in tumor progression remains unknown (119, 132). Recently, Maeda and coworkers showed that the manipulation of N-cadherin expression in NMuMG cells did not interfere with TGFβ-induced morphological changes (261). They were able to show that ablation of N-cadherin does not prevent the formation of stress fibers and focal adhesions in short term (1 day) TGFβ-treated NMuMG cells, indicating that N-cadherin is not required for the initiation of EMT. However, it was reported that N-cadherin depletion significantly reduced the migratory capability of epithelial NMuMG cells. There are several publications showing, that N-cadherin expression promotes motility and migration in different cell systems (118, 125, 286). Inconsistent with these findings we did not observe any significant alteration of the migratory ability of mesenchymal MTΔEcad and NMuMG LT cells upon N-cadherin depletion.

Given that the upregulation of N-cadherin during EMT is not an immediate early event but is increasing concomitantly with the downregulation of E-cadherin, depletion of N-cadherin during ongoing EMT or in mesenchymal cells, was performed to study its function and implication during this process. Although N-cadherin expression is increased in mesenchymal cells, we show that it is not required to maintain the mesenchymal state and that its attenuation does not induce the reverse mesenchymal to epithelial transition. Furthermore, the depletion of N-cadherin in epithelial cells was not sufficient to prevent TGFβ-induced EMT in NMuMG cells showing the characteristic regulation of epithelial and mesenchymal marker proteins and gain of a spindle-like cell shape. Interestingly, consistent with these findings, recent studies in our laboratory showed that forced expression of N-cadherin in the human mammary tumor cell line MCF7 did not induce EMT, keeping the cells in an epithelial state. (Dr. A. Kunita, unpublished data).

Former studies in our laboratory have identified N-cadherin as a component of the NCAM-mediated FGFR-4 signaling complex, which has been shown to stimulate β_1-integrin-mediated cell-matrix adhesion as well as the formation of cellular protrusions (133). Furthermore, it has been shown that neurite outgrowth but not cell-matrix adhesion is dependent on NCAM expression and the assembly of the FGFR-4 signaling complex in β-cells of pancreatic tumors (βT2-cells). Although being a component of the complex, our studies showed that N-cadherin is not essential for the formation of neurites in βT2-cells.

The function of N-cadherin during EMT is still ambiguous and may also be influenced by the cell type and the extracellular environment. Our studies show that N-cadherin is not a trigger of EMT and that its expression is not required to retain the mesenchymal state. It remains elusive, whether this is due to compensatory effects of other proteins. However, proteolytic processing of N-cadherin and subsequent release of its intracellular fragment (Ncad/CTF2) is thought to play an important role in the regulation of cell proliferation, invasion and differentiation (136). Ncad/CTF2 has been shown to interfere with gene transcription via the regulation of transcription complexes such as CBP/CREB by binding to CBP or TCF/LEF via the regulation of β-catenin (136, 137). Hence, it remains to be elucidated whether the loss of N-cadherin and its cleavage fragment Ncad/CTF2 have an impact on intracellular signaling and gene transcription and warrants further investigation.

6 Cancer stem cells - a potential outcome of EMT?

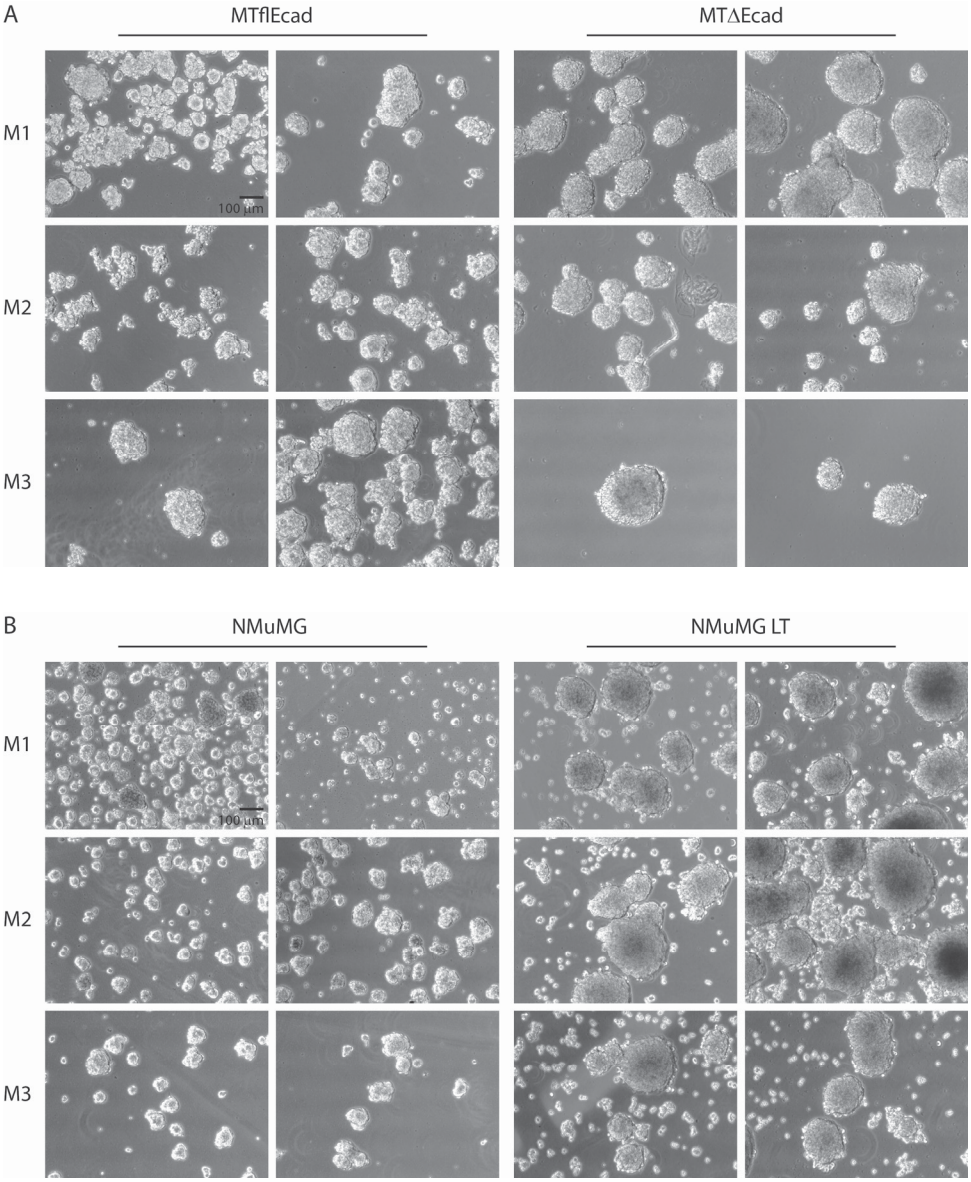
6.1 Results

6.1.1 Mesenchymal cells show increased sphere formation capacity

Due to hyperproliferation combined with genetic instability, advanced tumors show a high grade of heterogeneity regarding their differentiation state. Conclusive studies showed the existence of cancer stem cells, a new subclass of neoplastic cells within tumors. There is substantial evidence suggesting that EMT is associated with the generation of cancer stem cells (CSC), primarily due to its ability to change the differentiation state of cells (207, 214, 235, 236).

To investigate whether EMT results in the generation of a cancer stem cell subpopulation or increased stemness of the whole mesenchymal population we analyzed *in vitro* EMT systems for the gain of cancer stem cell properties. The three EMT cell systems used were the previously described epithelial MTflEcad cells with their mesenchymal counterparts MTΔEcad with Cre-mediated E-cadherin depletion as well as the epithelial NMuMG cells and NMuMG cells initially treated for more than 30 days with TGFβ (NMuMG LT). The third system used was the epithelial murine Py2T cells, an MMTV-PyMT (264) mammary tumor-derived cell line, and their mesenchymal analog initially treated for more than 30 days with TGFβ (Py2T LT) (generated by Lorenz Waldmeier, unpublished data). To assess whether the mesenchymal cells showed an alteration in stem cell properties we investigated their mammosphere formation capacity. The mammosphere formation assay is commonly used to analyze anchorage-independent growth of self renewing cancer stem cells. The ability to form mammospheres was found to be a characteristic of cancer stem cells and correlated with tumor initiating capacity (267). A common observation in all three cell systems was the increased ability of mesenchymal cells to form smooth, round and relatively large spheres, whereas the epithelial counterparts either formed clumpy aggregates or spheres of smaller size (Figure 30). Specifically, the MTflEcad formed spheres of various sizes and sowed increasing sphere forming capacity with increasing passages, indicating a selection for cells with anchorage independent growth competence. Whereas

the mesenchymal MTΔEcad cells grow as large round spheres with decreasing number of spheres in later passages (Figure 30A).



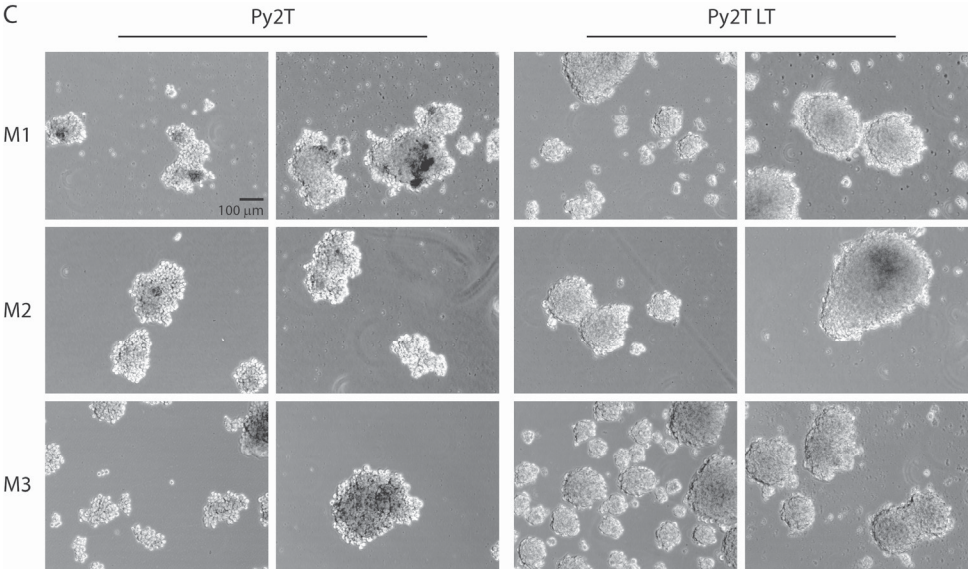


Figure 30: Mesenchymal cells show a higher tendency to form smooth mammospheres. Phase contrast images of mammospheres formed by (A) epithelial MTflEcad and mesenchymal MTΔEcad, (B) epithelial NMuMG and mesenchymal NMuMG LT and (C) epithelial Py2T and mesenchymal Py2T LT cells at indicated passages: first seeding (M1), first passaging (M2), second passaging (M3).

A similar tendency was found for the NMuMG cells, where the epithelial cells formed a high number of small spheres and the mesenchymal NMuMG LT cells generated large, smooth and spheric structures (Figure 30B). In contrast to these findings, the epithelial Py2T cells showed a poor sphere formation capacity, generating cell aggregates. The mesenchymal Py2T LT cells were, similar to other systems, able to form large, round mammospheres (Figure 30C). Due to the inability of the epithelial Py2T cells to form spheres, we did not further characterize the shape and structure of these bodies. In summary, mesenchymal cells show a higher capability to form large spheres indicating an increased anchorage-independent growth capacity and resistance to anoikis.

6.1.2 TGFβ-mediated EMT increases the resistance against anoikis

To assess the survival and/or proliferation capacity of epithelial and mesenchymal cells grown under anchorage independent conditions, spheres were disaggregated and the total cell number determined before reseeding. The TGFβ-induced EMT cell systems (NMuMG and Py2T) clearly showed increased survival of mesenchymal cells with increasing cell numbers throughout serial plating in comparison to their poorly proliferating epithelial counterparts (Figure 31B,C). In contrast, Cre-mediated E-cadherin ablation resulted in low

survival of the mesenchymal MTΔEcad cells compared to the proliferative epithelial cells (Figure 31A). From these data we can infer that TGFβ-induced EMT leads to increased resistance to anoikis and higher replicative potential upon serial passaging under anchorage-independent culturing conditions, suggesting a gain in self-renewal capacitiy.

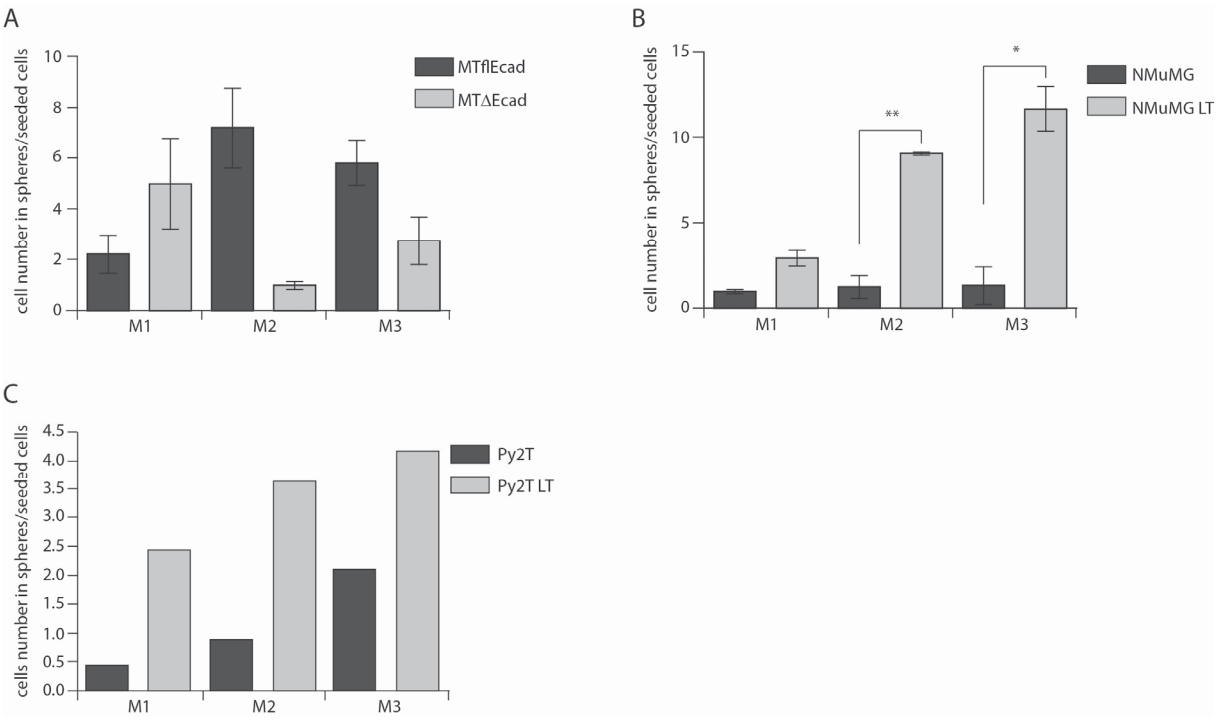


Figure 31: TGFβ-mediated EMT increases survival under anchorage independent growth conditions. Mammospheres generated from (A) MTflEcad and MTΔEcad, (B) NMuMG and NMuMG LT and (C) Py2T and Py2T LT cells were disaggregated after 7 days of culturing, and cell numbers were determined by counting at indicated passages, using a Neubauer chamber. Data are means of two independent experiments (A/B) and one single experiment (C) normalized to the number of initially seeded cells. Statistical values were calculated by using an unpaired two-tailed t-test. P-values ≤0.05 indicated with (*), p-values ≤ 0.01 indicated with (**).

To characterize the spheres formed by epithelial and mesenchymal cells, we firstly determined the sphere size. Recent publications showed that the ability to form larger mammospheres reflects progenitor cell proliferation and correlates with the potency to form tumors in severe combined immunodeficiency disease (SCID) mice (287, 288). Measurement of the mammosphere areas showed a significant increase in the average size of the mesenchymal cell-derived spheres (Figure 32C,D). While a significant difference was only seen in the capability to form large spheres in the MTflEcad / MTΔEcad system, the epithelial NMuMG cells nearly exclusively gave rise to small spheric structures and the

mesenchymal NMuMG LT cells primarily formed mainly medium-sized spheres (Figure 32A,B).

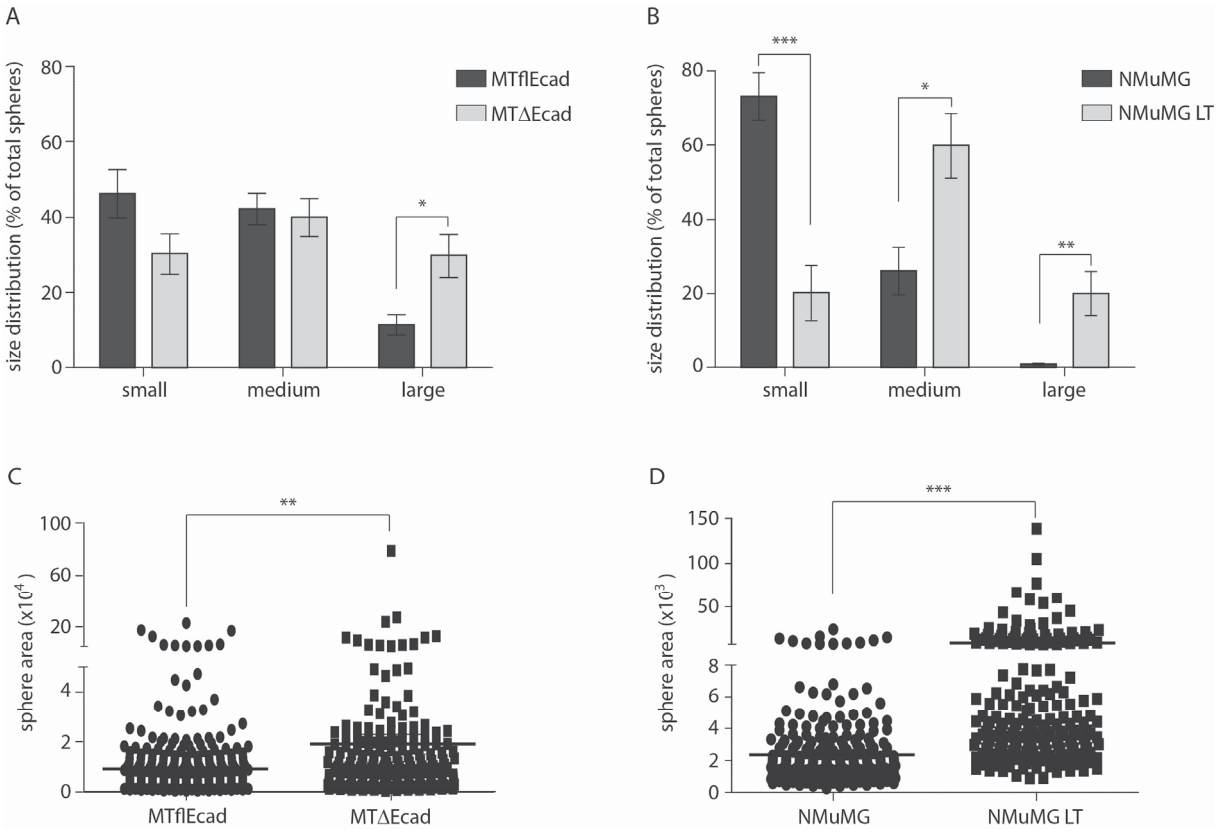


Figure 32: Mesenchymal cells give rise to significantly larger mammospheres. (A) Size distribution of spheres grown from MTflEcad and MTΔEcad cells was determined by measuring the area. Areas were divided in small ≤ 4'000 pixels (≡ 3365 µm^2), 4'000 pixels > medium ≤ 15'000 pixels (≡ 12'618 µm^2) and large > 15'000 pixels. Data is shown as percentage of total spheres counted and represents the mean of 3 independent experiments. Statistical values were calculated using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*).(B) Size distribution of spheres grown from NMuMG and NMuMG LT cells was determined by measuring the area. Areas were divided in small ≤ 2'000 pixels (≡ 1680 µm^2), 2'000 pixels > medium ≤ 15'000 pixels (≡ 12'618 µm^2) and large > 15'000 pixels. Data is shown as percentage of total spheres counted and represents the mean of 3 independent experiments. Statistical values were calculated using an unpaired two-tailed t-test. P-values ≤ 0.05 indicated with (*), p-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***). Sphere sizes of (C) MTflEcad (N=417) and MTΔEcad (N=227) and (D) NMuMG (N=299) and NMuMG LT (N=198) were measured in pixels. Statistical values were calculated using an unpaired two-tailed t-test. P-values ≤ 0.01 indicated with (**), p-values ≤ 0.001 indicated with (***).

We further characterized the spheres by determining the number of spheres formed by the epithelial and mesenchymal cells. We observed a significantly lower number of mammospheres generated by the MTΔEcad as well as the NMuMG LT cells compared to their respective epithelial counterparts (Figure 33). In summary, we could show that mesenchymal cells form less but larger and smoother spheres and TGFβ-mediated EMT clearly increases the proliferative capacity in anchorage-independent culturing.

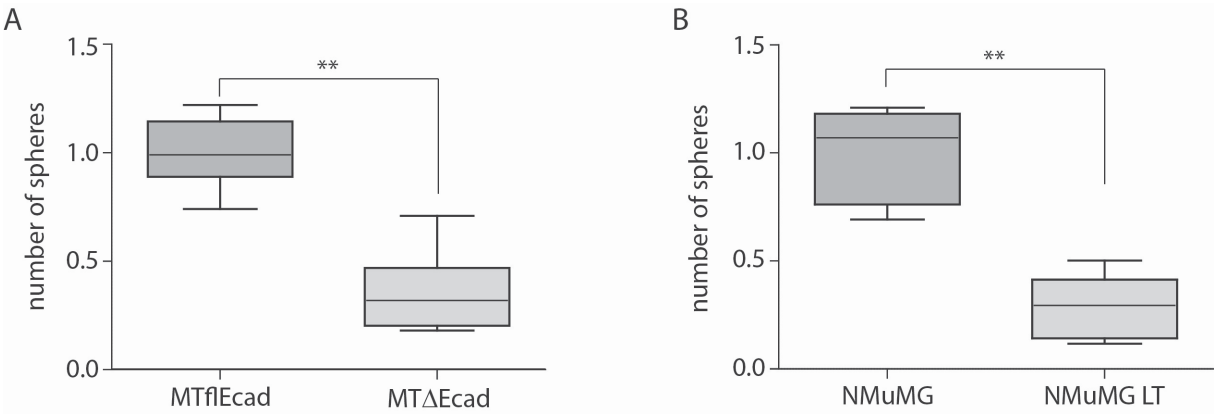


Figure 33: Mesenchymal cells form significantly less spheroids. (A) MTflEcad and MTΔEcad and (B) NMuMG and NMuMG LT cells were plated in triplicates in 96-well plates and mammospheres were counted after 3 days. Data represents values of two independent experiments normalized to the mean sphere numbers of epithelial MTflEcad cells. Statistical values were calculated using a paired two-tailed t-test. P-values ≤ 0.01 indicated with (**).

6.2 Cancer stem cell surface marker expression

One major requirement to make cancer stem cells accessible for drug targeting is the clear identification of these cells by defined markers. There are ongoing studies in the field to designate broadly applicable but specific surface marker proteins, and an important number of marker combinations have already been proposed for correlation with cancer stemness and tumorigenicity.

6.2.1 Hyaluronate receptor (CD44) is not an applicable murine cancer stem cell marker

One of the first and most broadly used marker combination in breast cancer is the expression of $CD44^{+}$ $CD24^{low/-}$, defining a subpopulation with increased tumor initiation capacity in limiting dilution experiments and elevated drug resistance (207, 223-225). Assessing the expression of this population during EMT revealed that cell surface expression of CD44 in NMuMG cells was positive, relatively uniform and independent of their epithelial or mesenchymal phenotype or culturing conditions (Figure 34A). However, the epithelial MTflEcad cells and their mesenchymal counterparts MTΔEcad showed very low levels of CD44 expression, which was again independent of the differentiation state (Figure 34B). In neither of our EMT cell systems were we able to detect a CD44-defined subpopulation, suggesting that this marker could be used to identify human cancer stem cells, but is not applicable for mouse cell systems.

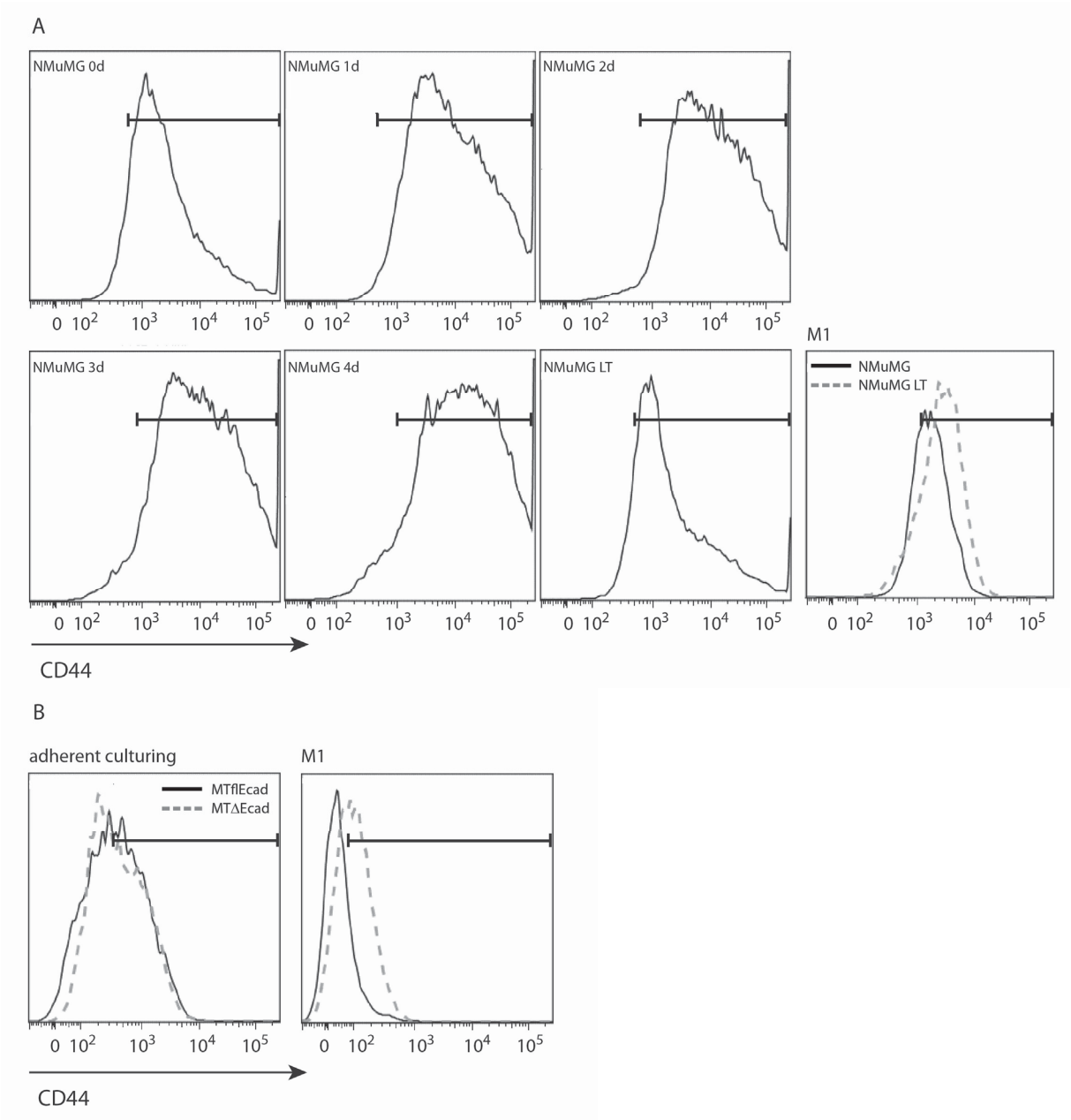
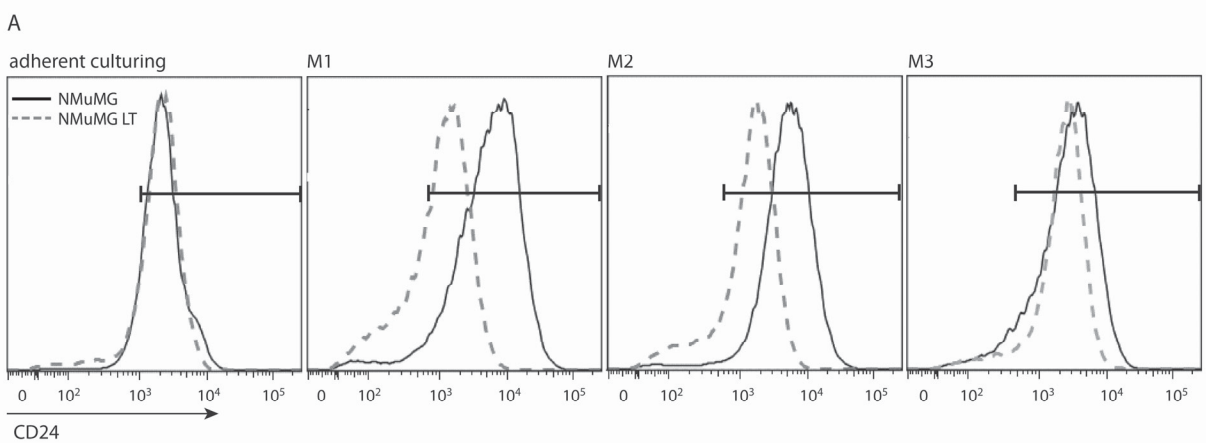


Figure 34: CD44 does not define a cancer stem cell subpopulation and does not increase with EMT. (A) FACS distribution of CD44-PE stained NMuMG cells treated with TGFβ for the indicated time under adherent culture conditions or cultured as mammospheres (M1). The gate represents the CD44-positive fraction (B) FACS distribution of CD44-PE stained MTflEcad and MTΔEcad cells either cultured under adherent or non-adherent (M1) conditions. The bar represents the CD44 positive fraction.

6.2.2 CD24 surface expression varies between different EMT cell systems

CD24 is a widely used marker in human and mouse breast cancer cell lines and tumors and is used in combination with various other markers, such as β_1-integrin, α_6-integrin, CD133 or Sca-1 to selectively define the cancer stem cell subpopulation (226, 227,

289, 290). Therefore, we investigated whether the expression of CD24 protein correlated with EMT or whether mammosphere culturing conditions selected for a CD24-defined subpopulation. FACS analysis of CD24-stained epithelial NMuMG and mesenchymal long term TGFβ treated (LT) cells showed that NMuMG cells expressed CD24 independently of their epithelial or mesenchymal state or culturing conditions (Figure 35). In mammosphere serial plating experiments we detected a slight decreased percentage of $CD24^+$ cells in M1 (significant) and M2 followed by an increase in M3. The decrease was due to a shift of the whole population and we were unable to define a CD24-specific subpopulation.

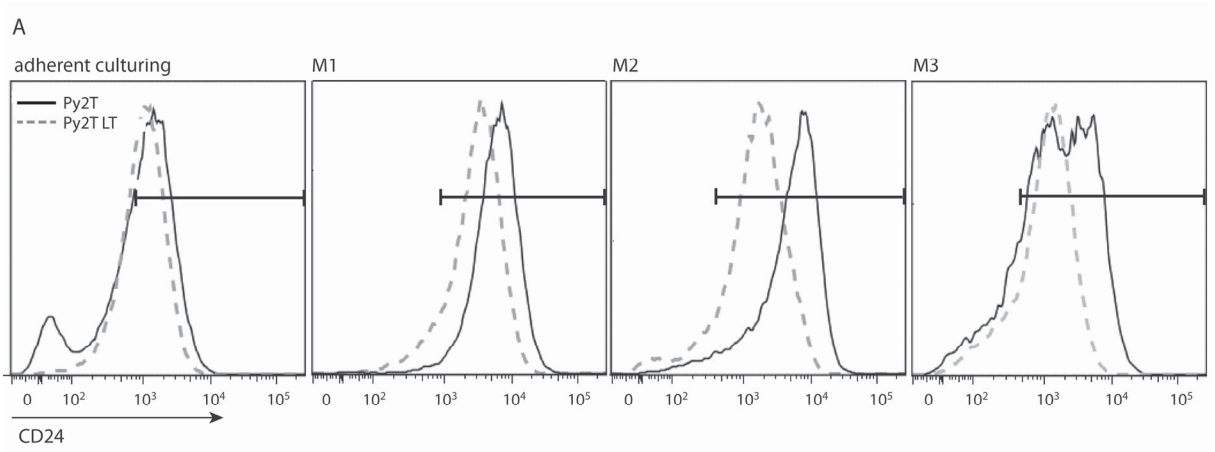


B

$CD24^+$	adherent (N=8)	M1 (N=2)	M2 (N=2)	M3 (N=2)
NMuMG	97.8 % (± 2.6)	99.1 % (± 0.4)	98.7 % (± 1.0)	97.6 % (± 1.6)
NMuMG LT	99.1 % (± 1.4)	91.7 % (± 1.8)	91.6 % (± 7.0)	99.1 % (± 0.9)

Figure 35: CD24 does not define a cancer stem cell subpopulation in NMuMG cells. (A) FACS analysis of CD24-stained NMuMG and NMuMG LT cells either cultured adherent or as mammospheres in serial passaging (M1-M3). The gate represents the CD24-positive fraction. Data were quantified in panel B. (B) Average percentage of $CD24^+$ stained cells (± SD) of adherent cultures or during serial replating of NMuMG and NMuMG LT mammospheres (M1-M3). In M1 NMuMG cells show a significantly higher CD24 staining (p-value = 0.0309) compared to NMuMG LT. Statistical values were calculated by using an unpaired two-tailed t-test.

Furthermore, we analyzed the second TGFβ-induced EMT cell line Py2T for the expression of CD24 and observed a comparable result as seen in the NMuMG cells (Figure 36). Epithelial Py2T and their mesenchymal counterparts Py2T LT showed a positive surface staining for CD24 with slightly decreased levels in the mesenchymal Py2T LT during passage M1 and M2 of the non-adherent cultured cells.

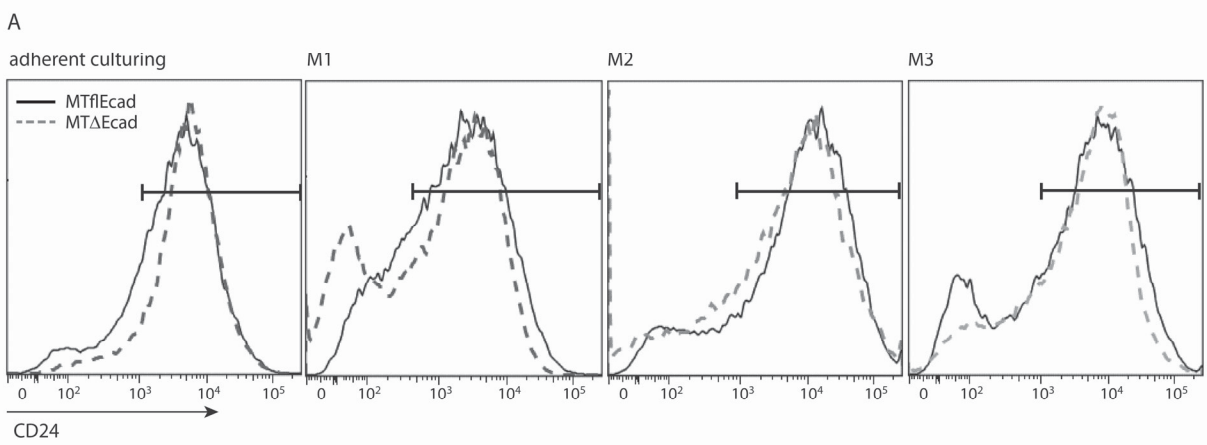


B

CD24⁺	adherent (N=3)	M1 (N=1)	M2 (N=1)	M3 (N=1)
Py2T	87.3 % (± 5.3)	98.7 %	90.1 %	68.2 %
Py2T LT	95.7 % (± 1.0)	95.1 %	79.8 %	64.1 %

Figure 36: Epithelial and mesenchymal Py2T and Py2T LT cells show a relatively uniform CD24 expression . (A) FACS analysis of cell surface expression of CD24 in adherent cultured or mammosphere serial passaging (M1-M3) of Py2T and Py2T LT cells. The gate represents the CD24-positive fraction. (B) Average percentage of $CD24^{+}$-stained cells (± SD) of adherent cultures or mammosphere serial replating (M1-M3) of Py2T and Py2T LT cells

Assessing the same surface marker expression in the epithelial MTflEcad compared to the mesenchymal MTΔEcad cells showed a high variability in the expression of CD24 (Figure 37). In this EMT cell system we observed a significant increase in the CD24-positive fraction in the mesenchymal MTΔEcad cells compared to their epithelial counterpart when the cells were cultured under adherent conditions. The same tendency was observed when the cells were cultured as mammospheres, though this culturing condition resulted in a high variability of the marker expression levels. These data indicate that CD24 cannot be used as a general cancer stem cell marker throughout different mouse mammary cell lines.



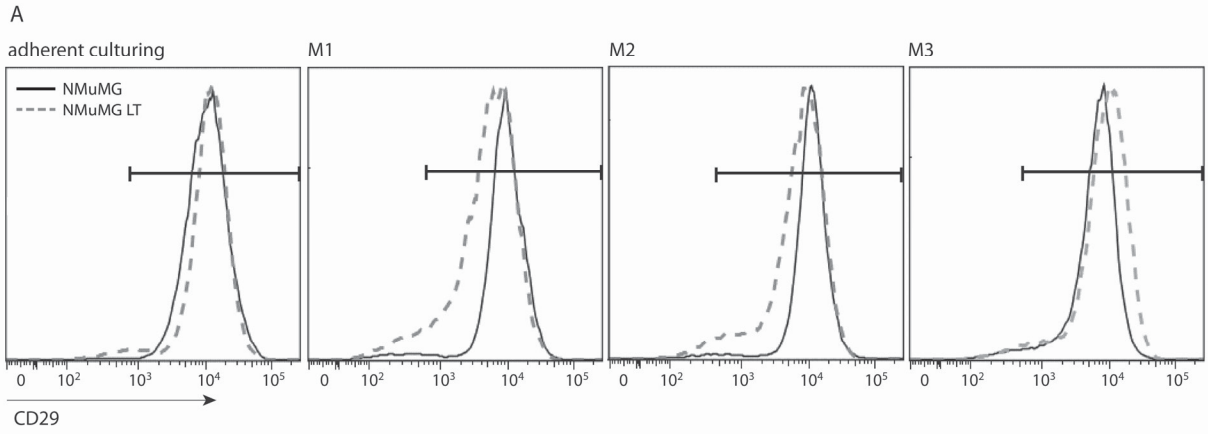
B

CD24⁺	adherent (N=7)	M1 (N=3)	M2 (N=2)	M3 (N=2)
MTflEcad	76.5 % (± 8.2)	63.1% (± 18.9)	51.5 % (± 43.9)	50.1 % (± 36.6)
MTΔEcad	94.3 % (± 3.2)	74.0 % (± 7.5)	78.1 % (± 10.0)	74.9 % (± 23.2)

Figure 37: Mesenchymal MTΔEcad cells show increased levels of CD24 compared to their epithelial counterparts. (A) FACS analysis of cell surface expression of CD24 in adherent cultured or mammosphere serial passaging (M1-M3) of MTflEcad and MTΔEcad cells. The gate represents the CD24-positive fraction. (B) Quantification of FACS data shown in (A) of $CD24^+$ stained cells (± SD) of adherent cultures or mammosphere serial replating (M1-M3) of MTflEcad and MTΔEcad cells. Adherent cultures of MTΔEcad cells show a significantly higher CD24 staining (p-value = 0.0002) compared to MTflEcad. Statistical values were calculated by using an unpaired two-tailed t-test.

6.2.3 CD29 shows uniformly high surface expression in EMT cell systems

In various studies high expression of β_1-integrin (CD29) in combination with medium to high expression of CD24 was shown to identify a subpopulation with increased tumor initiation capacity and cancer stem cell properties (229-231). We therefore investigated whether we were able to identify a CSC subpopulation in our EMT cell systems by assessing the surface expression of CD29. FACS analysis for the CD29 marker in NMuMG cells showed a uniformly high expression in the epithelial as well as in the mesenchymal cells independently of the culturing conditions (Figure 38).

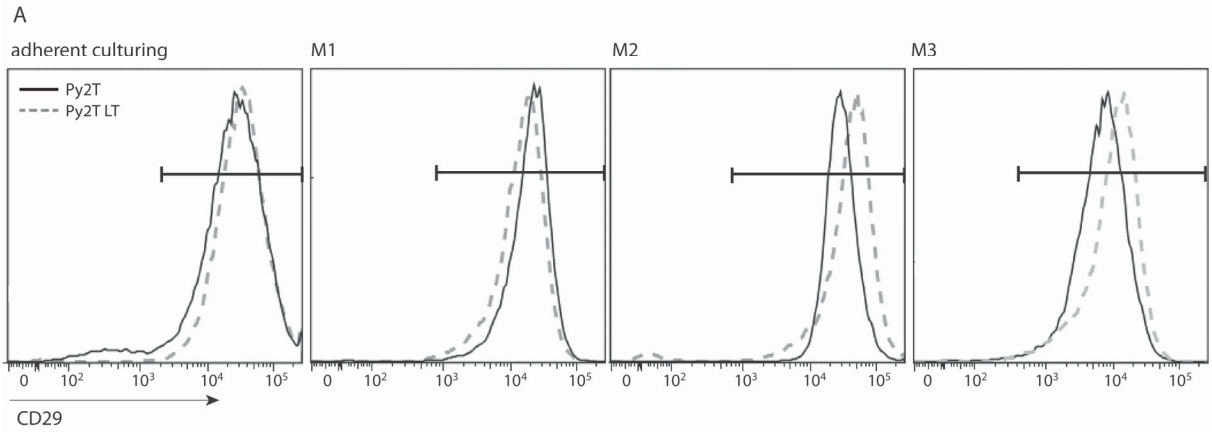


B

CD29⁺	adherent (N=7)	M1 (N=3)	M2 (N=2)	M3 (N=2)
NMuMG	99.9 % (± 0.1)	99.5 % (± 0.3)	99.3 % (± 0.9)	98.9 % (± 1.4)
NMuMG LT	99.8 % (± 0.2)	99.4 % (± 0.4)	99.6 % (± 0.1)	100 % (± 0)

Figure 38: NMuMG and NMuMG LT cells show a uniformly positive CD29 staining. (A) FACS analysis of CD29 surface expression in NMuMG and NMuMG LT cells either in adherent cultures or as mammospheres in serial passaging (M1-M3). The gate represents the CD29-positive fraction. Data was quantified in panel B. (B) Quantification of FACS data shown in (A) of $CD29^+$ stained cells (± SD) of NMuMG and NMuMG LT cells either adherent cultures or serial passages of mammosphere culturing.

Moreover, we observed the same expression pattern in Py2T cells, the second TGFβ-inducible EMT system, showing a high surface expression of CD29 in epithelial Py2T cells as well as in mesenchymal Py2T LT cells (Figure 39). In both cell systems we could not detect any discrete subpopulation defined by the expression of CD29.

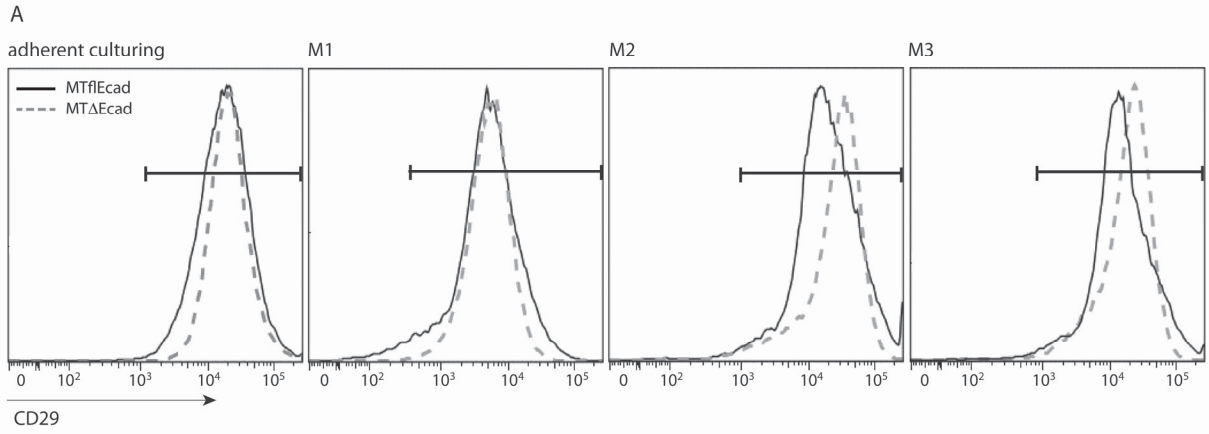


B

CD29⁺	adherent (N=3)	M1 (N=1)	M2 (N=1)	M3 (N=1)
Py2T	99.8 % (± 0.1)	100 %	99.9 %	99.9 %
Py2T LT	99.9 % (± 0.1)	100 %	99.8 %	100 %

Figure 39: Py2T and Py2T LT cells express high levels of CD29 on the cell surface. (A) FACS analysis of CD29 surface expression in Py2T and Py2T LT cells either cultured adherent or as mammospheres in serial passaging (M1-M3). The CD29 positive fraction is marked by a bar. Data was quantified in panel B. (B) Quantification of FACS data shown in (A) of $CD29^+$ stained cells (± SD) of Py2T and Py2T LT cells either adherent cultured or serial passages of mammosphere culturing.

Lastly, we investigated whether we were able to identify a cancer stem cell population by the expression of CD29 in the epithelial MTflEcad and its mesenchymal counterpart MTΔEcad. Consistent with the data found in the other cell systems we detected a high and uniform surface expression of CD29 independent of the differentiation state of the cells or the culturing conditions(Figure 40).



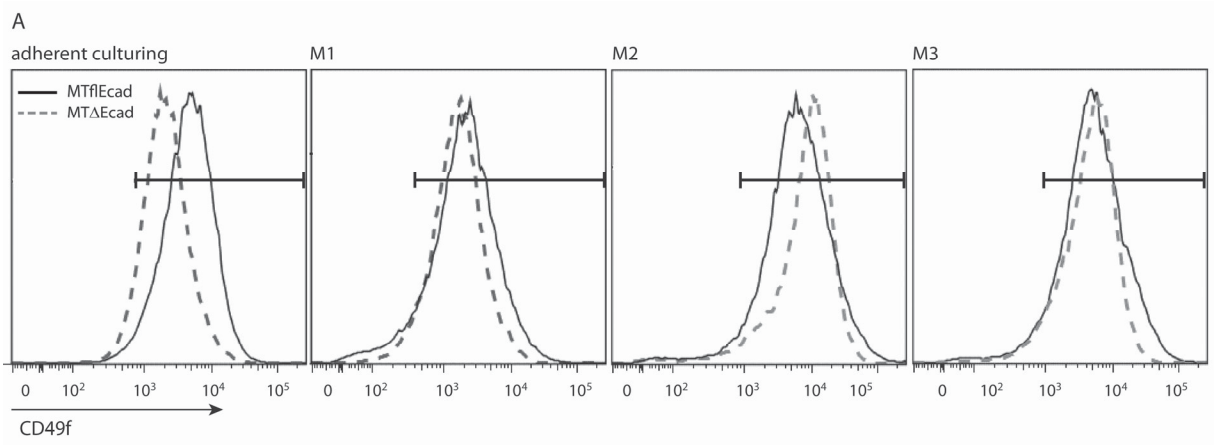
B

CD29⁺	adherent (N=7)	M1 (N=3)	M2 (N=2)	M3 (N=2)
MTflEcad	99.8 % (± 0.2)	99.7 % (± 0.5)	99.8 % (± 0.1)	99.9 % (± 0)
MTΔEcad	99.9 % (± 0.2)	99.9 % (± 0.2)	99.9 % (± 0.1)	99.9 % (± 0.1)

Figure 40: CD29 is uniformly expressed by MTflEcad and MTΔEcad cells. (A) FACS analysis of cell surface expression of CD29 in adherent cultured or mammosphere serial passaging (M1-M3) of MTflEcad and MTΔEcad cells. The bar represents positive CD29 staining and data is quantified in panel B.

6.2.4 CD49f is not expressed in all EMT systems

Several studies determined α_6-integrin (CD49f), another member of the integrin family, as a surface marker for cancer stem cells. It was demonstrated that a breast cancer cell subpopulation, sorted by $CD24^+/CD49f^{high}$ expression showed increased colony formation and enhanced tumor initiation capacity (226, 229). By FACS analysis of CD49f-stained epithelial and mesenchymal cells we assessed whether this surface marker identified a subpopulation or showed a difference in surface expression between epithelial and mesenchymal cells. we did not detect any expression of CD49f on the cell surface of epithelial NMuMG cells nor of mesenchymal NMuMG LT cells (data not shown). In contrast, the genetically-mediated EMT cell system MTflEcad / MTΔEcad showed a high surface expression of CD49f, which was of the same intensity in the epithelial and mesenchymal cells and independent of the culturing conditions (Figure 41). These data indicate that CD49f is not applicable to determine cancer stem cells in our EMT cell systems.



B

CD49f⁺	adherent (N=7)	M1 (N=3)	M2 (N=2)	M3 (N=2)
MTflEcad	96.8 % (± 2.0)	99.4 % (± 0.2)	99.5 % (± 0.4)	99.9 % (± 0.1)
MTΔEcad	98.6 % (± 1.1)	99.8 % (± 0.1)	99.7 % (± 0.3)	99.9 % (± 0.1)

Figure 41: CD49f is highly expressed on the surface of MTflEcad and MTΔEcad cells. (A) FACS analysis of cell surface expression of CD49f in adherent cultured or mammosphere serial passaging (M1-M3) of MTflEcad and MTΔEcad cells. The gate represents positive CD49f-staining and data is quantified in panel B.

6.3 Discussion

There is substantial evidence suggesting an association between cancer stem cells and a specific state of differentiation. It has indeed been demonstrated that the induction of EMT via the overexpression of the transcription factor Snail in trophoblast stem cells results in a metastable phenotype of preserved self-renewal and multipotency, and the loss of epithelial maintenance (236). Furthermore, it has been shown that the induction of EMT in transformed mammary epithelial cells generates a side-population enriched for CSC properties, such as increased tumorigenicity, mammosphere formation capacity, cell surface marker expression and increased drug-resistance (214, 238). These findings suggest a close connection between EMT and cancer stem cells and a better understanding of the biological processes leading to the generation and maintenance of CSCs might offer new perspectives into future cancer treatments. Thus, proving the coherence between EMT and cancer stem cells, as well as the identification of this population, is of major interest for ongoing research.

In the present study, we have assessed whether EMT increases stem cell properties by use of different *in vitro* EMT model systems. To address this question we analyzed epithelial and mesenchymal cells for their ability to form mammospheres. The mammosphere formation assay is well approved to assay anchorage-independent growth capacity. Sphere formation was found to be characteristic of cancer stem cells and correlates with tumor initiation capacity (267). Consistent with former findings, we demonstrated that mesenchymal cells when compared to their epithelial counterparts, showed an increased capacity to form smooth and large spheres, which has been previously reported to reflect progenitor cell proliferation and correlate with increased tumorigenicity (287, 288). Furthermore, we demonstrate increased proliferation of the mesenchymal cells of the TGFβ-induced EMT systems when cultured under anchorage-independent conditions, indicating that EMT mediates increased resistance to anoikis. Interestingly, consistent with these findings, recent studies in our laboratory have shown an increased tumor initiation capacity for the mesenchymal MTΔEcad cells compared with the epithelial MTflEcad cells (Dr. A. Fantozzi and D. Maaß, unpublished data).

Next, we wanted to assess whether EMT functions as a selection process for an underlying presence of cancer stem cells which would give rise to a defined CSC

subpopulation. An alternative consequence of EMT could be an increase in stem cell properties of the entire population due to the dedifferentiation process. Using FACS analysis for CSC markers this would either result in the appearance or an increase of a small subpopulation or in a shift of the entire population due to EMT. To answer this question we analyzed the three *in vitro* EMT cell systems for the expression of different surface markers, such as CD44, CD24, CD29, CD49f, which were formerly identified to define CSC subpopulations (223, 225-227, 229, 230, 289). Surprisingly, most markers tested were uniformly and highly expressed throughout all cell systems independently of the differentiation state of the cells. Consistent with former publications and recent tumor transplantation studies, only CD24 showed a significant increased surface expression in MTΔEcad cells compared to their epithelial counterparts, indicating increased CSC properties. Substantiation of this assumption warrants further investigation including sorting of the $CD24^{high}$ fraction and transplantation experiments of the sorted cells in mouse models. With the marker combinations tested, we were not able to define a discrete CSC subpopulation which would suggest an increase of cancer stem cell properties of the entire population as a consequence of EMT. Nevertheless, it remains unclear whether the subpopulation is non-existent or not detectable with the marker combinations used.

These data show that conclusive results are still lacking to define the connection between EMT and cancer stemness. The unavailability of general surface marker proteins to define cancer stem cells reveal the need for further research to define prevalent but specific CSC markers in order to make these cells accessible for therapy and by this to prevent patient relapse.

7 References

1. Hanahan D & Weinberg RA (2000) The hallmarks of cancer. *Cell* 100(1):57-70.
2. Hanahan D & Weinberg RA (2011) Hallmarks of cancer: the next generation. *Cell* 144(5):646-674.
3. Cheng N, Chytil A, Shyr Y, Joly A, & Moses HL (2008) Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. *Mol Cancer Res* 6(10):1521-1533.
4. Bhowmick NA, Neilson EG, & Moses HL (2004) Stromal fibroblasts in cancer initiation and progression. *Nature* 432(7015):332-337.
5. Fedi P, Tronick, S.R., and Aaronson, S.A. (1997) *Growth factors in cancer medicine* (Williams and Wilkins, Baltimore, MD).
6. Burkhart DL & Sage J (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* 8(9):671-682.
7. Curto M, Cole BK, Lallemand D, Liu CH, & McClatchey AI (2007) Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. *J Cell Biol* 177(5):893-903.
8. Partanen JI, Nieminen AI, & Klefstrom J (2009) 3D view to tumor suppression: Lkb1, polarity and the arrest of oncogenic c-Myc. *Cell Cycle* 8(5):716-724.
9. Adams JM & Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26(9):1324-1337.
10. Junttila MR & Evan GI (2009) p53--a Jack of all trades but master of none. *Nat Rev Cancer* 9(11):821-829.
11. Donehower LA*, et al.* (1995) Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. *Genes Dev* 9(7):882-895.
12. Amenta JS & Brocher SC (1981) Mechanisms of protein turnover in cultured cells. *Life Sci* 28(11):1195-1208.
13. Mizushima N (2007) Autophagy: process and function. *Genes Dev* 21(22):2861-2873.
14. Liang XH*, et al.* (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* 402(6762):672-676.
15. Degenhardt K*, et al.* (2006) Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* 10(1):51-64.
16. Baeriswyl V & Christofori G (2009) The angiogenic switch in carcinogenesis. *Semin Cancer Biol* 19(5):329-337.
17. Blasco MA (2005) Telomeres and human disease: ageing, cancer and beyond. *Nat Rev Genet* 6(8):611-622.
18. Kim NW*, et al.* (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science* 266(5193):2011-2015.
19. Cavallaro U & Christofori G (2004) Multitasking in tumor progression: signaling functions of cell adhesion molecules. *Ann N Y Acad Sci* 1014:58-66.
20. Nguyen DX & Massague J (2007) Genetic determinants of cancer metastasis. *Nat Rev Genet* 8(5):341-352.
21. Perou CM*, et al.* (2000) Molecular portraits of human breast tumours. *Nature* 406(6797):747-752.
22. Dairkee SH, Ljung BM, Smith H, & Hackett A (1987) Immunolocalization of a human basal epithelium specific keratin in benign and malignant breast disease. *Breast Cancer Res Treat* 10(1):11-20.
23. Rakha EA, Reis-Filho JS, & Ellis IO (2008) Basal-like breast cancer: a critical review. *J Clin Oncol* 26(15):2568-2581.
24. Reddy KB (2011) Triple-negative breast cancers: an updated review on treatment options. *Curr Oncol* 18(4):e173-179.
25. Gusterson B (2009) Do 'basal-like' breast cancers really exist? *Nat Rev Cancer* 9(2):128-134.
26. Carey LA*, et al.* (2006) Race, breast cancer subtypes, and survival in the Carolina Breast Cancer Study. *JAMA* 295(21):2492-2502.
27. Calza S*, et al.* (2006) Intrinsic molecular signature of breast cancer in a population-based cohort of 412 patients. *Breast Cancer Res* 8(4):R34.
28. Sorlie T*, et al.* (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* 98(19):10869-10874.
29. Wang Y*, et al.* (2005) Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. *Lancet* 365(9460):671-679.
30. Chi JT*, et al.* (2006) Gene expression programs in response to hypoxia: cell type specificity and prognostic significance in human cancers. *PLoS Med* 3(3):e47.
31. Hennessy BT*, et al.* (2009) Characterization of a naturally occurring breast cancer subset enriched in epithelial-to-mesenchymal transition and stem cell characteristics. *Cancer Res* 69(10):4116-4124.
32. Yilmaz M & Christofori G (2009) EMT, the cytoskeleton, and cancer cell invasion. *Cancer Metastasis Rev* 28(1-2):15-33.

33. Brabletz T, Jung A, Spaderna S, Hlubek F, & Kirchner T (2005) Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer* 5(9):744-749.
34. Lee JM, Dedhar S, Kalluri R, & Thompson EW (2006) The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 172(7):973-981.
35. Yilmaz M & Christofori G (2010) Mechanisms of motility in metastasizing cells. *Mol Cancer Res* 8(5):629-642.
36. Friedl P & Wolf K (2003) Tumour-cell invasion and migration: diversity and escape mechanisms. *Nat Rev Cancer* 3(5):362-374.
37. Christofori G (2006) New signals from the invasive front. *Nature* 441(7092):444-450.
38. De Wever O*, et al.* (2008) Molecular and pathological signatures of epithelial-mesenchymal transitions at the cancer invasion front. *Histochem Cell Biol* 130(3):481-494.
39. Thomas PA*, et al.* (1999) Association between keratin and vimentin expression, malignant phenotype, and survival in postmenopausal breast cancer patients. *Clin Cancer Res* 5(10):2698-2703.
40. Hlubek F*, et al.* (2007) Heterogeneous expression of Wnt/beta-catenin target genes within colorectal cancer. *Int J Cancer* 121(9):1941-1948.
41. Friedl P, Borgmann S, & Brocker EB (2001) Amoeboid leukocyte crawling through extracellular matrix: lessons from the Dictyostelium paradigm of cell movement. *J Leukoc Biol* 70(4):491-509.
42. Pankova K, Rosel D, Novotny M, & Brabek J (2010) The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. *Cell Mol Life Sci* 67(1):63-71.
43. Wolf K & Friedl P (2006) Molecular mechanisms of cancer cell invasion and plasticity. *Br J Dermatol* 154 Suppl 1:11-15.
44. Kerkela E & Saarialho-Kere U (2003) Matrix metalloproteinases in tumor progression: focus on basal and squamous cell skin cancer. *Exp Dermatol* 12(2):109-125.
45. Nabeshima K*, et al.* (2000) Front-cell-specific expression of membrane-type 1 matrix metalloproteinase and gelatinase A during cohort migration of colon carcinoma cells induced by hepatocyte growth factor/scatter factor. *Cancer Res* 60(13):3364-3369.
46. Hegerfeldt Y, Tusch M, Brocker EB, & Friedl P (2002) Collective cell movement in primary melanoma explants: plasticity of cell-cell interaction, beta1-integrin function, and migration strategies. *Cancer Res* 62(7):2125-2130.
47. Fidler IJ (1973) The relationship of embolic homogeneity, number, size and viability to the incidence of experimental metastasis. *Eur J Cancer* 9(3):223-227.
48. Friedl P & Wolf K (2008) Tube travel: the role of proteases in individual and collective cancer cell invasion. *Cancer Res* 68(18):7247-7249.
49. Wolf K*, et al.* (2007) Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nat Cell Biol* 9(8):893-904.
50. Wicki A*, et al.* (2006) Tumor invasion in the absence of epithelial-mesenchymal transition: podoplanin-mediated remodeling of the actin cytoskeleton. *Cancer Cell* 9(4):261-272.
51. Bonnomet A*, et al.* (2010) Epithelial-to-mesenchymal transitions and circulating tumor cells. *J Mammary Gland Biol Neoplasia* 15(2):261-273.
52. Labelle M*, et al.* (2008) Vascular endothelial cadherin promotes breast cancer progression via transforming growth factor beta signaling. *Cancer Res* 68(5):1388-1397.
53. Nierodzik ML & Karpatkin S (2006) Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell* 10(5):355-362.
54. Fidler IJ (2003) The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited. *Nat Rev Cancer* 3(6):453-458.
55. Tsuruo T & Fujita N (2008) Platelet aggregation in the formation of tumor metastasis. *Proc Jpn Acad Ser B Phys Biol Sci* 84(6):189-198.
56. Gupta GP*, et al.* (2007) Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature* 446(7137):765-770.
57. Minn AJ*, et al.* (2005) Genes that mediate breast cancer metastasis to lung. *Nature* 436(7050):518-524.
58. Weis SM & Cheresh DA (2005) Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 437(7058):497-504.
59. Muller A*, et al.* (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410(6824):50-56.
60. Kaplan RN, Rafii S, & Lyden D (2006) Preparing the "soil": the premetastatic niche. *Cancer Res* 66(23):11089-11093.
61. Kaplan RN*, et al.* (2005) VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 438(7069):820-827.
62. Hiratsuka S, Watanabe A, Aburatani H, & Maru Y (2006) Tumour-mediated upregulation of chemoattractants and recruitment of myeloid cells predetermines lung metastasis. *Nat Cell Biol* 8(12):1369-1375.
63. Erler JT*, et al.* (2009) Hypoxia-induced lysyl oxidase is a critical mediator of bone marrow cell recruitment to form the premetastatic niche. *Cancer Cell* 15(1):35-44.

64. Peinado H, Lavotshkin S, & Lyden D (2011) The secreted factors responsible for pre-metastatic niche formation: old sayings and new thoughts. *Semin Cancer Biol* 21(2):139-146.
65. Yang J & Weinberg RA (2008) Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell* 14(6):818-829.
66. Kalluri R (2009) EMT: when epithelial cells decide to become mesenchymal-like cells. *J Clin Invest* 119(6):1417-1419.
67. Kalluri R & Weinberg RA (2009) The basics of epithelial-mesenchymal transition. *J Clin Invest* 119(6):1420-1428.
68. Bloushtain-Qimron N*, et al.* (2008) Cell type-specific DNA methylation patterns in the human breast. *Proc Natl Acad Sci U S A* 105(37):14076-14081.
69. Shipitsin M*, et al.* (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* 11(3):259-273.
70. Graff JR, Gabrielson E, Fujii H, Baylin SB, & Herman JG (2000) Methylation patterns of the E-cadherin 5' CpG island are unstable and reflect the dynamic, heterogeneous loss of E-cadherin expression during metastatic progression. *J Biol Chem* 275(4):2727-2732.
71. Chaffer CL, Thompson EW, & Williams ED (2007) Mesenchymal to epithelial transition in development and disease. *Cells Tissues Organs* 185(1-3):7-19.
72. Polyak K & Weinberg RA (2009) Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nat Rev Cancer* 9(4):265-273.
73. Perez-Moreno M, Jamora C, & Fuchs E (2003) Sticky business: orchestrating cellular signals at adherens junctions. *Cell* 112(4):535-548.
74. Tsukita S, Furuse M, & Itoh M (2001) Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2(4):285-293.
75. Wheelock MJ & Johnson KR (2003) Cadherins as modulators of cellular phenotype. *Annu Rev Cell Dev Biol* 19:207-235.
76. Ireton RC*, et al.* (2002) A novel role for p120 catenin in E-cadherin function. *J Cell Biol* 159(3):465-476.
77. Davis MA, Ireton RC, & Reynolds AB (2003) A core function for p120-catenin in cadherin turnover. *J Cell Biol* 163(3):525-534.
78. Thoreson MA*, et al.* (2000) Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* 148(1):189-202.
79. Noren NK, Niessen CM, Gumbiner BM, & Burridge K (2001) Cadherin engagement regulates Rho family GTPases. *J Biol Chem* 276(36):33305-33308.
80. Wildenberg GA*, et al.* (2006) p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho. *Cell* 127(5):1027-1039.
81. Comoglio PM, Boccaccio C, & Trusolino L (2003) Interactions between growth factor receptors and adhesion molecules: breaking the rules. *Curr Opin Cell Biol* 15(5):565-571.
82. Park JI*, et al.* (2005) Kaiso/p120-catenin and TCF/beta-catenin complexes coordinately regulate canonical Wnt gene targets. *Dev Cell* 8(6):843-854.
83. Ferber EC*, et al.* (2008) A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus. *J Biol Chem* 283(19):12691-12700.
84. Thiery JP & Sleeman JP (2006) Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 7(2):131-142.
85. Zavadil J*, et al.* (2001) Genetic programs of epithelial cell plasticity directed by transforming growth factor-beta. *Proc Natl Acad Sci U S A* 98(12):6686-6691.
86. Savagner P, Yamada KM, & Thiery JP (1997) The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J Cell Biol* 137(6):1403-1419.
87. Lo HW*, et al.* (2007) Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. *Cancer Res* 67(19):9066-9076.
88. Birchmeier C & Gherardi E (1998) Developmental roles of HGF/SF and its receptor, the c-Met tyrosine kinase. *Trends Cell Biol* 8(10):404-410.
89. Herrlich P*, et al.* (1998) How tumor cells make use of CD44. *Cell Adhes Commun* 6(2-3):141-147.
90. Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P, & Ponta H (2002) CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* 16(23):3074-3086.
91. Gort EH, Groot AJ, van der Wall E, van Diest PJ, & Vooijs MA (2008) Hypoxic regulation of metastasis via hypoxia-inducible factors. *Curr Mol Med* 8(1):60-67.
92. Cannito S*, et al.* (2008) Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. *Carcinogenesis* 29(12):2267-2278.
93. Gregory PA*, et al.* (2008) The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* 10(5):593-601.
94. Park SM, Gaur AB, Lengyel E, & Peter ME (2008) The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. *Genes Dev* 22(7):894-907.
95. Warzecha CC, Sato TK, Nabet B, Hogenesch JB, & Carstens RP (2009) ESRP1 and ESRP2 are epithelial cell-type-specific regulators of FGFR2 splicing. *Mol Cell* 33(5):591-601.

96. Warzecha CC, Shen S, Xing Y, & Carstens RP (2009) The epithelial splicing factors ESRP1 and ESRP2 positively and negatively regulate diverse types of alternative splicing events. *RNA Biol* 6(5):546-562.
97. Xu J, Lamouille S, & Derynck R (2009) TGF-beta-induced epithelial to mesenchymal transition. *Cell Res* 19(2):156-172.
98. Christofori G (2003) Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression. *EMBO J* 22(10):2318-2323.
99. Bissell MJ & Radisky D (2001) Putting tumours in context. *Nat Rev Cancer* 1(1):46-54.
100. Schrader KA*, et al.* (2008) Hereditary diffuse gastric cancer: association with lobular breast cancer. *Fam Cancer* 7(1):73-82.
101. Berx G*, et al.* (1996) E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene* 13(9):1919-1925.
102. Cheng CW*, et al.* (2001) Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. *Oncogene* 20(29):3814-3823.
103. Peinado H, Portillo F, & Cano A (2004) Transcriptional regulation of cadherins during development and carcinogenesis. *Int J Dev Biol* 48(5-6):365-375.
104. Herranz N*, et al.* (2008) Polycomb complex 2 is required for E-cadherin repression by the Snail1 transcription factor. *Mol Cell Biol* 28(15):4772-4781.
105. Jenuwein T & Allis CD (2001) Translating the histone code. *Science* 293(5532):1074-1080.
106. Takeichi M (1993) Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 5(5):806-811.
107. Graff JR*, et al.* (1995) E-cadherin expression is silenced by DNA hypermethylation in human breast and prostate carcinomas. *Cancer Res* 55(22):5195-5199.
108. Peinado H, Olmeda D, & Cano A (2007) Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? *Nat Rev Cancer* 7(6):415-428.
109. Cano A*, et al.* (2000) The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2(2):76-83.
110. Comijn J*, et al.* (2001) The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 7(6):1267-1278.
111. Eger A*, et al.* (2005) DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. *Oncogene* 24(14):2375-2385.
112. Verstappen G*, et al.* (2008) Atypical Mowat-Wilson patient confirms the importance of the novel association between ZFHX1B/SIP1 and NuRD corepressor complex. *Hum Mol Genet* 17(8):1175-1183.
113. Perk J, Iavarone A, & Benezra R (2005) Id family of helix-loop-helix proteins in cancer. *Nat Rev Cancer* 5(8):603-614.
114. Zhu W, Leber B, & Andrews DW (2001) Cytoplasmic O-glycosylation prevents cell surface transport of E-cadherin during apoptosis. *EMBO J* 20(21):5999-6007.
115. Marambaud P*, et al.* (2002) A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. *EMBO J* 21(8):1948-1956.
116. Steinhusen U*, et al.* (2001) Cleavage and shedding of E-cadherin after induction of apoptosis. *J Biol Chem* 276(7):4972-4980.
117. Fujita Y*, et al.* (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat Cell Biol* 4(3):222-231.
118. Nieman MT, Prudoff RS, Johnson KR, & Wheelock MJ (1999) N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J Cell Biol* 147(3):631-644.
119. Hulit J*, et al.* (2007) N-cadherin signaling potentiates mammary tumor metastasis via enhanced extracellular signal-regulated kinase activation. *Cancer Res* 67(7):3106-3116.
120. Cavallaro U, Schaffhauser B, & Christofori G (2002) Cadherins and the tumour progression: is it all in a switch? *Cancer Lett* 176(2):123-128.
121. Birchmeier W & Behrens J (1994) Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1198(1):11-26.
122. Li G & Herlyn M (2000) Dynamics of intercellular communication during melanoma development. *Mol Med Today* 6(4):163-169.
123. Tomita K*, et al.* (2000) Cadherin switching in human prostate cancer progression. *Cancer Res* 60(13):3650-3654.
124. Hazan RB, Qiao R, Keren R, Badano I, & Suyama K (2004) Cadherin switch in tumor progression. *Ann N Y Acad Sci* 1014:155-163.
125. Islam S, Carey TE, Wolf GT, Wheelock MJ, & Johnson KR (1996) Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J Cell Biol* 135(6 Pt 1):1643-1654.
126. Li G, Satyamoorthy K, & Herlyn M (2001) N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res* 61(9):3819-3825.
127. Shintani Y, Maeda M, Chaika N, Johnson KR, & Wheelock MJ (2008) Collagen I promotes epithelial-to-mesenchymal transition in lung cancer cells via transforming growth factor-

beta signaling. *Am J Respir Cell Mol Biol* 38(1):95-104.

128. Alexander NR*, et al.* (2006) N-cadherin gene expression in prostate carcinoma is modulated by integrin-dependent nuclear translocation of Twist1. *Cancer Res* 66(7):3365-3369.
129. Theisen CS, Wahl JK, 3rd, Johnson KR, & Wheelock MJ (2007) NHERF links the N-cadherin/catenin complex to the platelet-derived growth factor receptor to modulate the actin cytoskeleton and regulate cell motility. *Mol Biol Cell* 18(4):1220-1232.
130. Heldin CH, Ostman A, & Ronnstrand L (1998) Signal transduction via platelet-derived growth factor receptors. *Biochim Biophys Acta* 1378(1):F79-113.
131. Kong D*, et al.* (2008) Platelet-derived growth factor-D overexpression contributes to epithelial-mesenchymal transition of PC3 prostate cancer cells. *Stem Cells* 26(6):1425-1435.
132. Cavallaro U & Christofori G (2004) Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nat Rev Cancer* 4(2):118-132.
133. Cavallaro U, Niedermeyer J, Fuxa M, & Christofori G (2001) N-CAM modulates tumour-cell adhesion to matrix by inducing FGF-receptor signalling. *Nat Cell Biol* 3(7):650-657.
134. Hazan RB, Phillips GR, Qiao RF, Norton L, & Aaronson SA (2000) Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. *J Cell Biol* 148(4):779-790.
135. Suyama K, Shapiro I, Guttman M, & Hazan RB (2002) A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell* 2(4):301-314.
136. Marambaud P*, et al.* (2003) A CBP binding transcriptional repressor produced by the PS1/epsilon-cleavage of N-cadherin is inhibited by PS1 FAD mutations. *Cell* 114(5):635-645.
137. Uemura K*, et al.* (2006) Activity-dependent regulation of beta-catenin via epsilon-cleavage of N-cadherin. *Biochem Biophys Res Commun* 345(3):951-958.
138. Tadokoro S*, et al.* (2003) Talin binding to integrin beta tails: a final common step in integrin activation. *Science* 302(5642):103-106.
139. Deryugina EI, Bourdon MA, Jungwirth K, Smith JW, & Strongin AY (2000) Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. *Int J Cancer* 86(1):15-23.
140. Mercurio AM & Rabinovitz I (2001) Towards a mechanistic understanding of tumor invasion--lessons from the alpha6beta 4 integrin. *Semin Cancer Biol* 11(2):129-141.
141. Legate KR, Montanez E, Kudlacek O, & Fassler R (2006) ILK, PINCH and parvin: the tIPP of integrin signalling. *Nat Rev Mol Cell Biol* 7(1):20-31.
142. Koenig A, Mueller C, Hasel C, Adler G, & Menke A (2006) Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells. *Cancer Res* 66(9):4662-4671.
143. Haraguchi M*, et al.* (2008) Snail regulates cell-matrix adhesion by regulation of the expression of integrins and basement membrane proteins. *J Biol Chem* 283(35):23514-23523.
144. Sharma M & Henderson BR (2007) IQ-domain GTPase-activating protein 1 regulates beta-catenin at membrane ruffles and its role in macropinocytosis of N-cadherin and adenomatous polyposis coli. *J Biol Chem* 282(11):8545-8556.
145. Cao J*, et al.* (2008) Membrane type 1 matrix metalloproteinase induces epithelial-to-mesenchymal transition in prostate cancer. *J Biol Chem* 283(10):6232-6240.
146. Ellerbroek SM, Wu YI, Overall CM, & Stack MS (2001) Functional interplay between type I collagen and cell surface matrix metalloproteinase activity. *J Biol Chem* 276(27):24833-24842.
147. Pulyaeva H*, et al.* (1997) MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. *Clin Exp Metastasis* 15(2):111-120.
148. Husemann Y*, et al.* (2008) Systemic spread is an early step in breast cancer. *Cancer Cell* 13(1):58-68.
149. Thiery JP, Acloque H, Huang RY, & Nieto MA (2009) Epithelial-mesenchymal transitions in development and disease. *Cell* 139(5):871-890.
150. Brabletz T*, et al.* (2001) Variable beta-catenin expression in colorectal cancers indicates tumor progression driven by the tumor environment. *Proc Natl Acad Sci U S A* 98(18):10356-10361.
151. Teschendorff AE, Journee M, Absil PA, Sepulchre R, & Caldas C (2007) Elucidating the altered transcriptional programs in breast cancer using independent component analysis. *PLoS Comput Biol* 3(8):e161.
152. Mani SA*, et al.* (2007) Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc Natl Acad Sci U S A* 104(24):10069-10074.
153. Dumont N*, et al.* (2008) Sustained induction of epithelial to mesenchymal transition activates DNA methylation of genes silenced in basal-like breast cancers. *Proc Natl Acad Sci U S A* 105(39):14867-14872.
154. Lien HC*, et al.* (2007) Molecular signatures of metaplastic carcinoma of the breast by large-scale transcriptional profiling: identification of genes potentially related to epithelial-mesenchymal transition. *Oncogene* 26(57):7859-7871.

155. Lecharpentier A*, et al.* (2011) Detection of circulating tumour cells with a hybrid (epithelial/mesenchymal) phenotype in patients with metastatic non-small cell lung cancer. *Br J Cancer*.
156. Hou JM*, et al.* (2011) Circulating tumor cells as a window on metastasis biology in lung cancer. *Am J Pathol* 178(3):989-996.
157. Valdes F*, et al.* (2002) The epithelial mesenchymal transition confers resistance to the apoptotic effects of transforming growth factor Beta in fetal rat hepatocytes. *Mol Cancer Res* 1(1):68-78.
158. Puisieux A, Valsesia-Wittmann S, & Ansieau S (2006) A twist for survival and cancer progression. *Br J Cancer* 94(1):13-17.
159. Ansieau S*, et al.* (2008) Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell* 14(1):79-89.
160. Yang AD*, et al.* (2006) Chronic oxaliplatin resistance induces epithelial-to-mesenchymal transition in colorectal cancer cell lines. *Clin Cancer Res* 12(14 Pt 1):4147-4153.
161. Kajiyama H*, et al.* (2007) Chemoresistance to paclitaxel induces epithelial-mesenchymal transition and enhances metastatic potential for epithelial ovarian carcinoma cells. *Int J Oncol* 31(2):277-283.
162. Li QQ*, et al.* (2009) Twist1-mediated adriamycin-induced epithelial-mesenchymal transition relates to multidrug resistance and invasive potential in breast cancer cells. *Clin Cancer Res* 15(8):2657-2665.
163. Cochrane DR, Spoelstra NS, Howe EN, Nordeen SK, & Richer JK (2009) MicroRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents. *Mol Cancer Ther* 8(5):1055-1066.
164. Knutson KL*, et al.* (2006) Immunoediting of cancers may lead to epithelial to mesenchymal transition. *J Immunol* 177(3):1526-1533.
165. Moody SE*, et al.* (2002) Conditional activation of Neu in the mammary epithelium of transgenic mice results in reversible pulmonary metastasis. *Cancer Cell* 2(6):451-461.
166. Kudo-Saito C, Shirako H, Takeuchi T, & Kawakami Y (2009) Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell* 15(3):195-206.
167. Massague J, Blain SW, & Lo RS (2000) TGFbeta signaling in growth control, cancer, and heritable disorders. *Cell* 103(2):295-309.
168. Massague J (2008) TGFbeta in Cancer. *Cell* 134(2):215-230.
169. Massague J (1998) TGF-beta signal transduction. *Annu Rev Biochem* 67:753-791.
170. Massague J (2000) How cells read TGF-beta signals. *Nat Rev Mol Cell Biol* 1(3):169-178.
171. Itoh S, Itoh F, Goumans MJ, & Ten Dijke P (2000) Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem* 267(24):6954-6967.
172. Moustakas A, Souchelnytskyi S, & Heldin CH (2001) Smad regulation in TGF-beta signal transduction. *J Cell Sci* 114(Pt 24):4359-4369.
173. Itoh S*, et al.* (1998) Transforming growth factor beta1 induces nuclear export of inhibitory Smad7. *J Biol Chem* 273(44):29195-29201.
174. Ebisawa T*, et al.* (2001) Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. *J Biol Chem* 276(16):12477-12480.
175. Engel ME, McDonnell MA, Law BK, & Moses HL (1999) Interdependent SMAD and JNK signaling in transforming growth factor-beta-mediated transcription. *J Biol Chem* 274(52):37413-37420.
176. Yu L, Hebert MC, & Zhang YE (2002) TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. *EMBO J* 21(14):3749-3759.
177. Leivonen SK, Hakkinen L, Liu D, & Kahari VM (2005) Smad3 and extracellular signal-regulated kinase 1/2 coordinately mediate transforming growth factor-beta-induced expression of connective tissue growth factor in human fibroblasts. *J Invest Dermatol* 124(6):1162-1169.
178. Kretzschmar M, Doody J, Timokhina I, & Massague J (1999) A mechanism of repression of TGFbeta/ Smad signaling by oncogenic Ras. *Genes Dev* 13(7):804-816.
179. Lu T, Tian L, Han Y, Vogelbaum M, & Stark GR (2007) Dose-dependent cross-talk between the transforming growth factor-beta and interleukin-1 signaling pathways. *Proc Natl Acad Sci U S A* 104(11):4365-4370.
180. Yamashita M*, et al.* (2008) TRAF6 mediates Smad-independent activation of JNK and p38 by TGF-beta. *Mol Cell* 31(6):918-924.
181. Bakin AV, Tomlinson AK, Bhowmick NA, Moses HL, & Arteaga CL (2000) Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. *J Biol Chem* 275(47):36803-36810.
182. Vinals F & Pouyssegur J (2001) Transforming growth factor beta1 (TGF-beta1) promotes endothelial cell survival during in vitro angiogenesis via an autocrine mechanism implicating TGF-alpha signaling. *Mol Cell Biol* 21(21):7218-7230.
183. Kim BC (2008) FoxO3a mediates transforming growth factor-beta1-induced apoptosis in FaO rat hepatoma cells. *BMB Rep* 41(10):728-732.
184. Conery AR*, et al.* (2004) Akt interacts directly with Smad3 to regulate the sensitivity to TGF-beta induced apoptosis. *Nat Cell Biol* 6(4):366-372.

185. Jang CW*, et al.* (2002) TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. *Nat Cell Biol* 4(1):51-58.
186. Takekawa M*, et al.* (2002) Smad-dependent GADD45beta expression mediates delayed activation of p38 MAP kinase by TGF-beta. *EMBO J* 21(23):6473-6482.
187. Ohgushi M*, et al.* (2005) Transforming growth factor beta-dependent sequential activation of Smad, Bim, and caspase-9 mediates physiological apoptosis in gastric epithelial cells. *Mol Cell Biol* 25(22):10017-10028.
188. Hoshino Y, Katsuno Y, Ehata S, & Miyazono K (2011) Autocrine TGF-beta protects breast cancer cells from apoptosis through reduction of BH3-only protein, Bim. *J Biochem* 149(1):55-65.
189. Ramjaun AR, Tomlinson S, Eddaoudi A, & Downward J (2007) Upregulation of two BH3-only proteins, Bmf and Bim, during TGF beta-induced apoptosis. *Oncogene* 26(7):970-981.
190. Blokzijl A*, et al.* (2003) Cross-talk between the Notch and TGF-beta signaling pathways mediated by interaction of the Notch intracellular domain with Smad3. *J Cell Biol* 163(4):723-728.
191. Zhang YE (2009) Non-Smad pathways in TGF-beta signaling. *Cell Res* 19(1):128-139.
192. Murillo MM, del Castillo G, Sanchez A, Fernandez M, & Fabregat I (2005) Involvement of EGF receptor and c-Src in the survival signals induced by TGF-beta1 in hepatocytes. *Oncogene* 24(28):4580-4587.
193. Grandis JR & Sok JC (2004) Signaling through the epidermal growth factor receptor during the development of malignancy. *Pharmacol Ther* 102(1):37-46.
194. Buck E*, et al.* (2007) Loss of homotypic cell adhesion by epithelial-mesenchymal transition or mutation limits sensitivity to epidermal growth factor receptor inhibition. *Mol Cancer Ther* 6(2):532-541.
195. Frederick BA*, et al.* (2007) Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma. *Mol Cancer Ther* 6(6):1683-1691.
196. Barr S*, et al.* (2008) Bypassing cellular EGF receptor dependence through epithelial-to-mesenchymal-like transitions. *Clin Exp Metastasis* 25(6):685-693.
197. Levy L & Hill CS (2006) Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. *Cytokine Growth Factor Rev* 17(1-2):41-58.
198. Bornstein S*, et al.* (2009) Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. *J Clin Invest* 119(11):3408-3419.
199. Ikushima H & Miyazono K (2010) TGFbeta signalling: a complex web in cancer progression. *Nat Rev Cancer* 10(6):415-424.
200. Gal A*, et al.* (2008) Sustained TGF beta exposure suppresses Smad and non-Smad signalling in mammary epithelial cells, leading to EMT and inhibition of growth arrest and apoptosis. *Oncogene* 27(9):1218-1230.
201. Takaku K*, et al.* (1998) Intestinal tumorigenesis in compound mutant mice of both Dpc4 (Smad4) and Apc genes. *Cell* 92(5):645-656.
202. Engle SJ*, et al.* (2002) Elimination of colon cancer in germ-free transforming growth factor beta 1-deficient mice. *Cancer Res* 62(22):6362-6366.
203. De Wever O & Mareel M (2003) Role of tissue stroma in cancer cell invasion. *J Pathol* 200(4):429-447.
204. Sanchez-Elsner T*, et al.* (2001) Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. *J Biol Chem* 276(42):38527-38535.
205. Padua D*, et al.* (2008) TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. *Cell* 133(1):66-77.
206. Gomis RR, Alarcon C, Nadal C, Van Poznak C, & Massague J (2006) C/EBPbeta at the core of the TGFbeta cytostatic response and its evasion in metastatic breast cancer cells. *Cancer Cell* 10(3):203-214.
207. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, & Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 100(7):3983-3988.
208. Cho RW & Clarke MF (2008) Recent advances in cancer stem cells. *Curr Opin Genet Dev* 18(1):48-53.
209. Bonnet D & Dick JE (1997) Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 3(7):730-737.
210. Gilbertson RJ & Rich JN (2007) Making a tumour's bed: glioblastoma stem cells and the vascular niche. *Nat Rev Cancer* 7(10):733-736.
211. Singh SK*, et al.* (2004) Identification of human brain tumour initiating cells. *Nature* 432(7015):396-401.
212. Li C*, et al.* (2007) Identification of pancreatic cancer stem cells. *Cancer Res* 67(3):1030-1037.
213. Beltran AS*, et al.* (2011) Generation of tumor initiating cells by exogenous delivery of OCT4 Transcription Factor. *Breast Cancer Res* 13(5):R94.
214. Mani SA*, et al.* (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell* 133(4):704-715.
215. Pontier SM & Muller WJ (2009) Integrins in mammary-stem-cell biology and breast-cancer

progression--a role in cancer stem cells? *J Cell Sci* 122(Pt 2):207-214.
216. Guo W & Giancotti FG (2004) Integrin signalling during tumour progression. *Nat Rev Mol Cell Biol* 5(10):816-826.
217. Guo W*, et al.* (2006) Beta 4 integrin amplifies ErbB2 signaling to promote mammary tumorigenesis. *Cell* 126(3):489-502.
218. Campos LS (2005) Beta1 integrins and neural stem cells: making sense of the extracellular environment. *Bioessays* 27(7):698-707.
219. Watt FM (2002) Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J* 21(15):3919-3926.
220. Taddei I*, et al.* (2008) Beta1 integrin deletion from the basal compartment of the mammary epithelium affects stem cells. *Nat Cell Biol* 10(6):716-722.
221. Costello RT*, et al.* (2000) Human acute myeloid leukemia CD34+/CD38- progenitor cells have decreased sensitivity to chemotherapy and Fas-induced apoptosis, reduced immunogenicity, and impaired dendritic cell transformation capacities. *Cancer Res* 60(16):4403-4411.
222. Bao S*, et al.* (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. *Nature* 444(7120):756-760.
223. Sheridan C*, et al.* (2006) CD44+/CD24- breast cancer cells exhibit enhanced invasive properties: an early step necessary for metastasis. *Breast Cancer Res* 8(5):R59.
224. Ponti D*, et al.* (2005) Isolation and in vitro propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 65(13):5506-5511.
225. Phillips TM, McBride WH, & Pajonk F (2006) The response of CD24(-/low)/CD44+ breast cancer-initiating cells to radiation. *J Natl Cancer Inst* 98(24):1777-1785.
226. Stingl J*, et al.* (2006) Purification and unique properties of mammary epithelial stem cells. *Nature* 439(7079):993-997.
227. Shackleton M*, et al.* (2006) Generation of a functional mammary gland from a single stem cell. *Nature* 439(7072):84-88.
228. Sleeman KE*, et al.* (2007) Dissociation of estrogen receptor expression and in vivo stem cell activity in the mammary gland. *J Cell Biol* 176(1):19-26.
229. Vassilopoulos A*, et al.* (2008) Identification and characterization of cancer initiating cells from BRCA1 related mammary tumors using markers for normal mammary stem cells. *Int J Biol Sci* 4(3):133-142.
230. Shafee N*, et al.* (2008) Cancer stem cells contribute to cisplatin resistance in Brca1/p53-mediated mouse mammary tumors. *Cancer Res* 68(9):3243-3250.
231. Zhang M*, et al.* (2008) Identification of tumor-initiating cells in a p53-null mouse model of breast cancer. *Cancer Res* 68(12):4674-4682.
232. Bidlingmaier S, Zhu X, & Liu B (2008) The utility and limitations of glycosylated human CD133 epitopes in defining cancer stem cells. *J Mol Med (Berl)* 86(9):1025-1032.
233. Silva IA*, et al.* (2011) Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. *Cancer Res* 71(11):3991-4001.
234. Park SY*, et al.* (2010) Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. *Clin Cancer Res* 16(3):876-887.
235. Ullmann U*, et al.* (2007) Epithelial-mesenchymal transition process in human embryonic stem cells cultured in feeder-free conditions. *Mol Hum Reprod* 13(1):21-32.
236. Jordan NV, Johnson GL, & Abell AN (2011) Tracking the intermediate stages of epithelial-mesenchymal transition in epithelial stem cells and cancer. *Cell Cycle* 10(17):2865-2873.
237. Morel AP*, et al.* (2008) Generation of breast cancer stem cells through epithelial-mesenchymal transition. *PLoS One* 3(8):e2888.
238. Asiedu MK, Ingle JN, Behrens MD, Radisky DC, & Knutson KL (2011) TGFbeta/TNF(alpha)-mediated epithelial-mesenchymal transition generates breast cancer stem cells with a claudin-low phenotype. *Cancer Res* 71(13):4707-4719.
239. Bao B*, et al.* (2011) Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells. *J Cell Biochem* 112(9):2296-2306.
240. Wellner U*, et al.* (2009) The EMT-activator ZEB1 promotes tumorigenicity by repressing stemness-inhibiting microRNAs. *Nat Cell Biol* 11(12):1487-1495.
241. Prat A*, et al.* (2010) Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res* 12(5):R68.
242. Kaufmann E & Knochel W (1996) Five years on the wings of fork head. *Mech Dev* 57(1):3-20.
243. Kaestner KH, Knochel W, & Martinez DE (2000) Unified nomenclature for the winged helix/forkhead transcription factors. *Genes Dev* 14(2):142-146.
244. Wotton KR & Shimeld SM (2011) Analysis of lamprey clustered Fox genes: Insight into Fox gene evolution and expression in vertebrates. *Gene* 489(1):30-40.
245. Fuxe J, Vincent T, & Garcia de Herreros A (2010) Transcriptional crosstalk between TGF-beta and stem cell pathways in tumor cell invasion: role of EMT promoting Smad complexes. *Cell Cycle* 9(12):2363-2374.
246. Taube JH*, et al.* (2010) Core epithelial-to-mesenchymal transition interactome gene-expression signature is associated with

claudin-low and metaplastic breast cancer subtypes. *Proc Natl Acad Sci U S A* 107(35):15449-15454.

247. Wu WX*, et al.* (2009) [Relationship between epithelial-mesenchymal transition and basal cell-like phenotype in breast cancer]. *Zhonghua Bing Li Xue Za Zhi* 38(8):519-523.
248. Fu Z & Tindall DJ (2008) FOXOs, cancer and regulation of apoptosis. *Oncogene* 27(16):2312-2319.
249. Aitola M, Carlsson P, Mahlapuu M, Enerback S, & Pelto-Huikko M (2000) Forkhead transcription factor FoxF2 is expressed in mesodermal tissues involved in epithelio-mesenchymal interactions. *Dev Dyn* 218(1):136-149.
250. Blixt A*, et al.* (1998) The two-exon gene of the human forkhead transcription factor FREAC-2 (FKHL6) is located at 6p25.3. *Genomics* 53(3):387-390.
251. Hellqvist M, Mahlapuu M, Blixt A, Enerback S, & Carlsson P (1998) The human forkhead protein FREAC-2 contains two functionally redundant activation domains and interacts with TBP and TFIIB. *J Biol Chem* 273(36):23335-23343.
252. Varambally S*, et al.* (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* 419(6907):624-629.
253. Lan Y & Jiang R (2009) Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 136(8):1387-1396.
254. Katoh Y & Katoh M (2008) Hedgehog signaling, epithelial-to-mesenchymal transition and miRNA (review). *Int J Mol Med* 22(3):271-275.
255. Yu W, Ruest LB, & Svoboda KK (2009) Regulation of epithelial-mesenchymal transition in palatal fusion. *Exp Biol Med (Maywood)* 234(5):483-491.
256. Wilkie AO & Morriss-Kay GM (2001) Genetics of craniofacial development and malformation. *Nat Rev Genet* 2(6):458-468.
257. Wang T*, et al.* (2003) Forkhead transcription factor Foxf2 (LUN)-deficient mice exhibit abnormal development of secondary palate. *Dev Biol* 259(1):83-94.
258. Ormestad M*, et al.* (2006) Foxf1 and Foxf2 control murine gut development by limiting mesenchymal Wnt signaling and promoting extracellular matrix production. *Development* 133(5):833-843.
259. Yang Z*, et al.* (2010) The mouse forkhead gene Foxp2 modulates expression of the lung genes. *Life Sci* 87(1-2):17-25.
260. Shi W*, et al.* (2011) MicroRNA-301 mediates proliferation and invasion in human breast cancer. *Cancer Res* 71(8):2926-2937.
261. Maeda M, Johnson KR, & Wheelock MJ (2005) Cadherin switching: essential for behavioral but not morphological changes during an epithelium-to-mesenchyme transition. *J Cell Sci* 118(Pt 5):873-887.
262. Lehembre F*, et al.* (2008) NCAM-induced focal adhesion assembly: a functional switch upon loss of E-cadherin. *EMBO J* 27(19):2603-2615.
263. Deckers M*, et al.* (2006) The tumor suppressor Smad4 is required for transforming growth factor beta-induced epithelial to mesenchymal transition and bone metastasis of breast cancer cells. *Cancer Res* 66(4):2202-2209.
264. Guy CT, Cardiff RD, & Muller WJ (1992) Induction of mammary tumors by expression of polyomavirus middle T oncogene: a transgenic mouse model for metastatic disease. *Mol Cell Biol* 12(3):954-961.
265. Hellqvist M, Mahlapuu M, Samuelsson L, Enerback S, & Carlsson P (1996) Differential activation of lung-specific genes by two forkhead proteins, FREAC-1 and FREAC-2. *J Biol Chem* 271(8):4482-4490.
266. Weber M*, et al.* (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat Genet* 39(4):457-466.
267. Dontu G*, et al.* (2003) In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. *Genes Dev* 17(10):1253-1270.
268. Duss S*, et al.* (2007) An oestrogen-dependent model of breast cancer created by transformation of normal human mammary epithelial cells. *Breast Cancer Res* 9(3):R38.
269. van 't Veer LJ*, et al.* (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 415(6871):530-536.
270. Yilmaz M (2009) The role of the cell adhesion molecule NCAM and the transcription factor Dlx2 in epithelial-mesenchymal transition (EMT) and tumor progression. Inauguraldissertation (der Universität Basel, Basel).
271. Muller WJ, Sinn E, Pattengale PK, Wallace R, & Leder P (1988) Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell* 54(1):105-115.
272. Derksen PW*, et al.* (2006) Somatic inactivation of E-cadherin and p53 in mice leads to metastatic lobular mammary carcinoma through induction of anoikis resistance and angiogenesis. *Cancer Cell* 10(5):437-449.
273. Piek E, Moustakas A, Kurisaki A, Heldin CH, & ten Dijke P (1999) TGF-(beta) type I receptor/ALK-5 and Smad proteins mediate epithelial to mesenchymal transdifferentiation in NMuMG breast epithelial cells. *J Cell Sci* 112 (Pt 24):4557-4568.
274. Verschueren K*, et al.* (1999) SIP1, a novel zinc finger/homeodomain repressor, interacts with Smad proteins and binds to 5'-CACCT sequences in candidate target genes. *J Biol Chem* 274(29):20489-20498.

275. Dohadwala M*, et al.* (2006) Cyclooxygenase-2-dependent regulation of E-cadherin: prostaglandin E(2) induces transcriptional repressors ZEB1 and snail in non-small cell lung cancer. *Cancer Res* 66(10):5338-5345.
276. Norton JD, Deed RW, Craggs G, & Sablitzky F (1998) Id helix-loop-helix proteins in cell growth and differentiation. *Trends Cell Biol* 8(2):58-65.
277. Kondo M*, et al.* (2004) A role for Id in the regulation of TGF-beta-induced epithelial-mesenchymal transdifferentiation. *Cell Death Differ* 11(10):1092-1101.
278. Derynck R, Akhurst RJ, & Balmain A (2001) TGF-beta signaling in tumor suppression and cancer progression. *Nat Genet* 29(2):117-129.
279. Fabregat I*, et al.* (2000) Epidermal growth factor impairs the cytochrome C/caspase-3 apoptotic pathway induced by transforming growth factor beta in rat fetal hepatocytes via a phosphoinositide 3-kinase-dependent pathway. *Hepatology* 32(3):528-535.
280. Lee MK*, et al.* (2007) TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J* 26(17):3957-3967.
281. Willis SN*, et al.* (2005) Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* 19(11):1294-1305.
282. Kim JY, Ahn HJ, Ryu JH, Suk K, & Park JH (2004) BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. *J Exp Med* 199(1):113-124.
283. Ploner C, Kofler R, & Villunger A (2009) Noxa: at the tip of the balance between life and death. *Oncogene* 27:S84-S92.
284. Qin Y, Capaldo C, Gumbiner BM, & Macara IG (2005) The mammalian Scribble polarity protein regulates epithelial cell adhesion and migration through E-cadherin. *J Cell Biol* 171(6):1061-1071.
285. Frixen UH*, et al.* (1991) E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J Cell Biol* 113(1):173-185.
286. Hazan RB, Kang L, Whooley BP, & Borgen PI (1997) N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. *Cell Adhes Commun* 4(6):399-411.
287. Grimshaw MJ*, et al.* (2008) Mammosphere culture of metastatic breast cancer cells enriches for tumorigenic breast cancer cells. *Breast Cancer Res* 10(3):R52.
288. Liu S*, et al.* (2008) BRCA1 regulates human mammary stem/progenitor cell fate. *Proc Natl Acad Sci U S A* 105(5):1680-1685.
289. Wright MH*, et al.* (2008) Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. *Breast Cancer Res* 10(1):R10.
290. Grange C, Lanzardo S, Cavallo F, Camussi G, & Bussolati B (2008) Sca-1 identifies the tumor-initiating cells in mammary tumors of BALB-neuT transgenic mice. *Neoplasia* 10(12):1433-1443.

8 Curriculum vitae

Name:	**Heck** Chantal Beatrice
Date of Birth:	09 November 1979
Place of Birth:	Buochs, NW
Nationality:	Swiss

Education:	01/2012	PhD examination
	2006 - 2011 **PhD thesis**	University of Basel Department of Biomedicine Institute of Biochemistry and Genetics Supervisor: Prof. Dr. Gerhard Christofori
	04/2006	Diploma examinations
	2004 - 2005 **Diploma thesis**	University of Zurich Department of Biochemistry, Zurich Supervisor: Prof. Dr. Andreas Plückthun
	2003 - 2005 **Advanced study period**	Biochemistry at the University of Zurich
	2000 - 2003 **Basic study period**	Biochemistry/Chemistry at the University of Zurich
	1992 - 1999 **Gymnasium**	Kollegium St. Fidelis, Stans

Conferences

December 5-7, 2007	MetaBre/BRECOSM breast cancer and metastasis conference Rome, Italy Oral presentation
September 21, 2010	BioValley Science Day Basel, Switzerland Poster
September 20, 2011	BioValley Science Day Basel, Switzerland Poster

Awards

BioValley Poster Award 2011

Publications

Spano, D., Heck, C., De Antonellis, P., Christofori, G. and Zollo, M., (2012) Molecular networks that regulate cancer metastasis. Sem. Cancer Biol., in revision.

Heck, C., Yilmaz, M., Meyer-Schaller, N. and Christofori, G., (2012) A pro-apoptotic and morphogenic function of Foxf2 during TGFβ-induced EMT. (manuscript in preparation)

Fantozzi, A., Maaß, D., Heck, C., Meyer-Schaller, N., Hopfer, U., Kunita, A. and Christofori, G., (2012) EMT and cancer stem cells: VEGFA induced angiogenesis, the missing link to tumor initiation. (manuscript in preparation)

9 Acknowledgments

First and foremost I would like to thank my supervisor Prof. Dr. Gerhard Christofori for giving me the opportunity to work on the exciting topics described in this thesis. I am grateful for his guidance, support and especially for the encouragement over the past five years. I always had the freedom to follow my ideas and to extend my scientific experience.

I also wish to thank the following people for their help to complete this work:

- Dorothea Maaß for our discussions, which were always inspiring and motivating. But I also want to thank her for hundreds of cheerful coffee breaks and a precious friendship.
- Dr. Nathalie Meyer-Schaller for her support and lots of helpful contributions to my project such as methods, background knowledge and scientific writing. And of course I enjoyed a lot the neighborhood and the constructive discussions.
- Dr. Natalie Schlegel for all the support at work as well as in private affairs. It was always helpful to discuss problems or questions with her as she was always lending a helping hand and offering useful inputs.
- Dr. Anna Fantozzi and Lorenz Waldmeier for great support and helpful discussions.
- Petra Schmidt for all her help in experiments and a very cordially nature.
- Dr. Ernesta Fagiani and Dr. Vanessa Baeriswyl for helpful support in FACS analysis and stainings as well as for many cheerful and happy moments.
- Maren Diepenbruck and Dr. Akiko Kunita for hilarious moments and all the members of the Christofori group for a pleasant, inspiring and friendly atmosphere in the lab and for countless unforgettable moments.

Finally I would like to thank my family, who supported me during the time of my study and thesis. They always gave me encouragement and never lost trust in me. I thank my beloved husband Markus for his love, patience, his preserving, constructive support and for thousands of other reasons.