The E- and N-cadherin switch in Epithelial to Mesenchymal Transition and metastasis
Potential drug targets?

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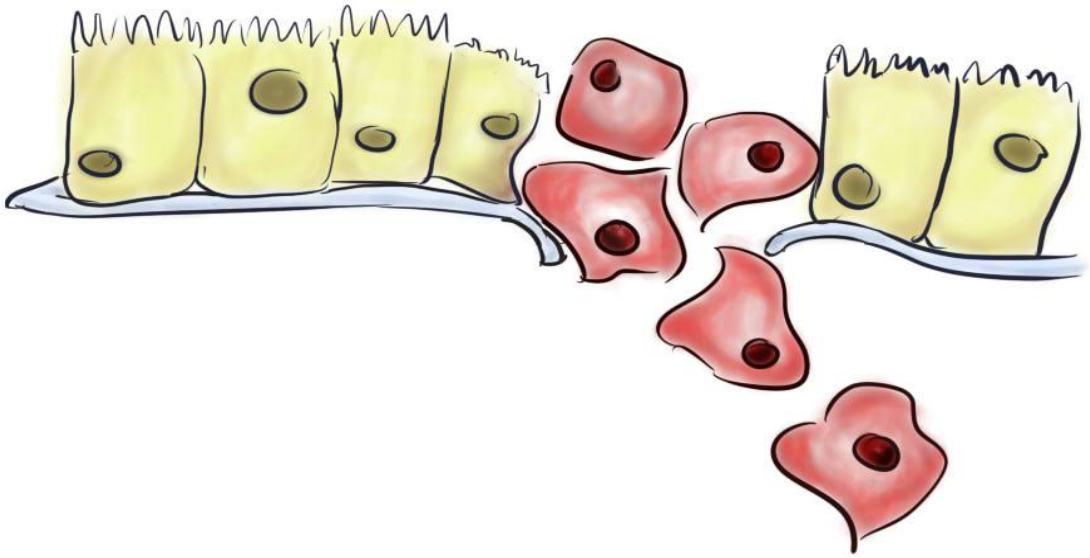

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Foreword

This thesis or writing assignment was written to complete my master Cancer Genomics and Developmental Biology at Utrecht University. The thesis is focused on E- to N-cadherin switch in Epithelial to Mesenchymal Transition during metastasis and the development of new targeted treatments.

Layman's summary

Carcinomas or epithelial cancers are the most prevalent type of cancer. In epithelial tissues, such as the skin, cells are connected very closely to each other. Epithelial to Mesenchymal Transition (EMT) is a cellular program which enables epithelial cells to break free from neighboring epithelial cells, partially through downregulation of epithelial cell-cell adhesion proteins. EMT is an important process in the development of animals. However, EMT can also be used by epithelial cancer cells to break free from the primary cancer. EMT in carcinomas is correlated with a higher number of metastasis and a lower survival. EMT can create mesenchymal cancer cells with a fibroblastic phenotype and a stem cell signature. During EMT, E-cadherin is often downregulated and N-cadherin is often upregulated, this is called the E- to N-cadherin switch. Cadherins are cell-cell adhesion proteins. Many carcinomas show the E- to N-cadherin switch, however, there are also other cadherin switches and E- and N-cadherin can be expressed simultaneously in several tissues and carcinomas. E-cadherin generally inhibits invasion via epithelial cell-cell adhesion. N-cadherin promotes invasion, through regulation of cell-cell adhesions of invading cells. Loss of E-cadherin might lead to EMT in both mouse models and cancer cell lines, however, there are many exceptions. In the future, new anticancer therapies might target EMT through upregulation of E-cadherin or by targeting N-cadherin. Targeting EMT might be beneficial as an anticancer strategy, because mesenchymal cancer stem cells are more resistant to chemotherapy than other epithelial cancer cells. Through combination of EMT inhibitors and chemotherapy, chemotherapy might be more efficient. This would lead to less metastases and recurrences and a higher patient survival.

Abstract

Carcinomas are most prevalent type of cancers and arise from an epithelial layer. Epithelial layers have a strict organization; cells are tightly linked through different junctions. The Epithelial to Mesenchymal Transition (EMT) developmental program enables epithelial cells to transform into mesenchymal cells and break free from neighboring epithelial cells in order to migrate though the body. Also in carcinomas, EMT enables cells to invade into healthy tissue. EMT also aids cancer progression in other manners by suppressing senescence, anoikis, apoptosis, oncogene addiction and modulating the immune response. Furthermore, EMT can lead to the formation of mesenchymal cancer stem cells. Mesenchymal cancer stem cells seem to be more resistant to chemotherapy than epithelial cancer cells. All of these traits increase the chance of an invading cancer cell being successful in setting up a metastatic niche. The cadherin switch from E-cadherin to N-cadherin is considered a mark of EMT. Cadherins are cell-cell adhesion proteins. E-cadherin inhibits invasion by protecting epithelial integrity and N-cadherin increases invasion. It seems that a part of carcinomas has an E- to N-cadherin switch during EMT. However, other carcinomas show other cadherin switches and E- and N-cadherin expression is not always mutually exclusive. In the HMLE cancer cell line E-cadherin knockdown is enough to induce EMT. By knocking out E-cadherin and p53 in a tissue specific manner they show an increase of invasion and metastases. In gastric carcinoma model E-cadherin and p53 knockout leads to EMT, however, in an invasive lobular carcinoma model EMT has not been observed. If EMT could be inhibited in carcinomas, the chance of metastasis would decrease and the formation of new cancer stem cells would be inhibited. Since chemotherapy mainly targets epithelial cancer cells, combining an EMT inhibitor might lead to more efficient chemotherapy. There are some compounds capable of inhibiting EMT or promoting the reverse process Mesenchymal to Epithelial Transition (MET). The identified compounds either inhibit a pathway which induces EMT or lead regaining epithelial identity and re-expression of E-cadherin. These compounds need to be further tested using *in vivo* models. Antibodies targeting mesenchymal marker proteins are being developed as well. An anti N-cadherin antibody shows a decrease of tumor growth and metastases in mice, perhaps by targeting mesenchymal cancer stem cells. Since chemotherapy does not efficiently target cancer stem cells, screens were performed to find compounds that target cancer stem cells. In the future, there may be a combined anticancer therapy developed which inhibits EMT and targets cancer stem cells. This type of therapy could be combined with chemo-, radiotherapy or surgery to treat carcinomas more efficiently.

Introduction

Cancer is one of the leading causes of death in the western world. Primary cancers often seed metastases. These metastases are most harmful and are the cause of death in around 90% of the cancer patients (Mehlen & Puisieux 2006). Epithelial cancers or carcinomas are the most common type of malignant neoplasms in the Netherlands (www.cijfersoverkanker.nl, 2013). The onset to cancer can be initiated with a genetic mutations causing cells to start proliferating abnormally, leading to a hyperplasia. However, just enabling proliferation is not enough. Normally when a cell acquires a mutation that enables proliferation, such as $BRAF^{V600E}$, the daughter cells go into oncogene induced senescence after a number of divisions (Peeper 2011). Oncogene induced senescence is a state in which cells inhibit proliferation persistently. To become cancerous, a cell needs to obtain the ability to proliferate indefinitely, evade growth suppressors, resist cell death, enable angiogenesis and metastasize (Hanahan & Weinberg 2011). To metastasize an epithelial cancer cell needs to be able to delaminate from the epithelial formation, break through the basal lamina, migrate through tissue, inhibit anoikis, invade the bloodstream, extravasate a blood vessel and set up a metastatic niche in a distant tissue (Geiger & Peeper 2009). Anoikis is apoptosis through lack of or inappropriate anchorage (Geiger & Peeper 2009). Epithelial cells are often linked closely to each other through cell-cell adhesion, such as desmosomes, focal adhesion junctions, tight junctions, adherens junctions and gap junctions. The epithelial cell-cell adhesions make it difficult for cells to break free from the epithelial layer.

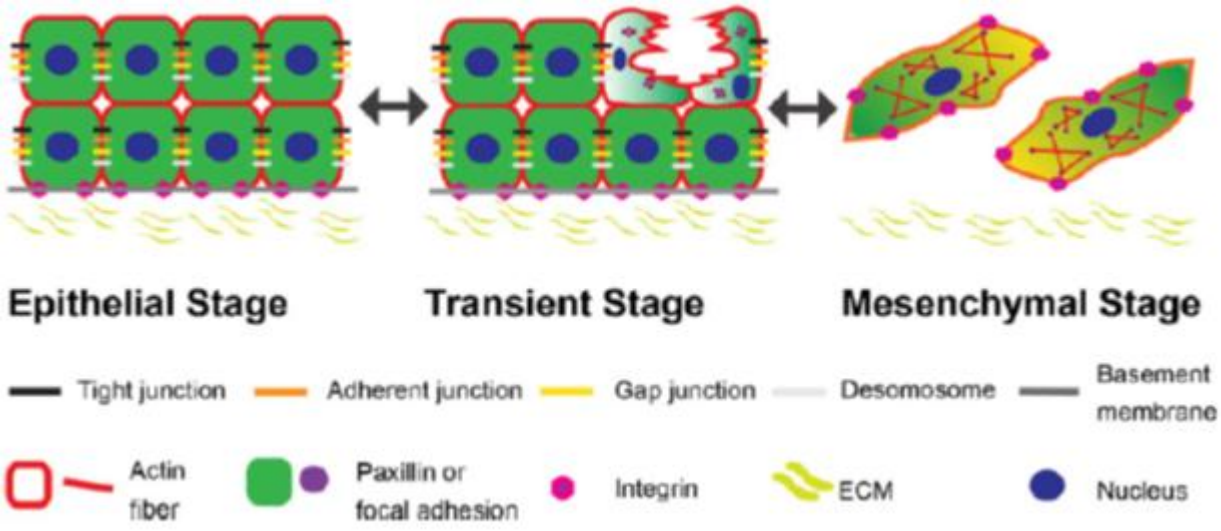

Fig. 1: Different stages of EMT. Cells can break from the epithelial organization through EMT. Cells in the epithelial layer are tightly linked through desmosomes, focal adhesions, tight junctions, adherence junctions and gap junctions. Epithelial cells show cortical actin and are linked to the extracellular matrix and basement membrane though integrins. Mesenchymal cells show actin stress fibers and a fibroblastic morphology. Additionally, during EMT, cells can acquire a stem cell like state and cortical actin is replaced by actin stress fibers. Adapted from Tiwari *et al*. 2012.

Table 1: Proteins involved in EMT (Tiwari *et al.* 2012; Yilmaz *et al.* 2007; Kalluri & Weinberg 2009; Sánchez-Tilló *et al.* 2012)

Epithelial marker	**Gene name**	**Function**
E-cadherin	CDH1	Adherens junction component
Zonula occludens-1	ZO-1	Tight junction component
Desmoplakin	DSP	Desmosomal component
Keratins		Intermediate filament

Mesenchymal marker	**Gene name**	**Function**
N-cadherin	CDH2	Cell adhesion in adherens junctions
Vimentin	VIM	Intermediate filament
Fibronectin	FM1	Extracellular matrix component
Metalloproteases	MMP -2, -3, -9	Extracellular matrix remodeling
Zeb1 and -2		EMT transcription factor
Twist1		EMT transcription factor
Snail1 and -2		EMT transcription factor

Epithelial to Mesenchymal Transition (EMT) is a developmental program that enables epithelial cells to lower adhesion to the epithelial layer, break through the basal lamina and migrate through mesenchymal tissue (Baum *et al.* 2008). EMT is an evolutionary conserved mechanism, which has been described in model organisms such as *C. elegans, Drosophila* and Sea Urchins (Baum *et al.* 2008). In mice EMT is an important process in development, playing a role in the primitive streak, cardiogenesis and somatogenesis (Nakagawa & Takeichi 1995; Takeichi 1988; Takeichi 1995). EMT is thought to play an important role in the process of metastasis of epithelial cancers (Tiwari *et al.* 2012; Sánchez-Tilló *et al.* 2012). Additionally, EMT can be a survival mechanism for cancer cells, via inhibition of apoptosis, anoikis, relieve oncogene addiction. Furthermore, EMT leads to an increased chemo resistance and immunosuppression (Tiwari *et al.* 2012). During EMT, proteins involved in epithelial cell adhesion are downregulated and proteins involved in migrating through mesenchymal tissue are upregulated (Fig. 1). Proteins that are often downregulated during EMT are E-cadherin, ZO-1, Desmoplakin and Cytokeratins. Other proteins are upregulated during EMT. For example: N-cadherin, vimentin, fibronectin, metalloproteases, α5β6 integrin, smooth muscle actin and nuclear β-catenin (Tiwari *et al.* 2012). The functions of these proteins are addressed in Table 1. Additionally, the actin network changes form cortical actin to actin stress fibers during EMT (Haynes *et al.* 2011) and cancer cells positive for mesenchymal marker proteins are more invasive *in vivo* (Patsialou *et al.* 2012; Shimada *et al.* 2012). EMT produces cells with a fibroblastic phenotype (Yilmaz *et al.* 2007) and a stem cell signature (Mani *et al.* 2008). Although, EMT and stem cellness might not always be coupled, depending on which EMT transcription factor is involved (Ocaña *et al.* 2012). Expression of EMT transcription factors Twist and Snail1 leads to an EMT with stem cell signature (Mani *et al.* 2008), though expression of EMT transcription factor Prrx1 suppresses stem cellness during EMT (Ocaña *et al.* 2012). Cancer stem cells fuel the tumor (Schepers *et al.* 2012; Chen *et al.* 2013). Patients with carcinomas that have gone through EMT have a lower survival rate, perhaps due to mesenchymal cancer stem cells being more resilient to

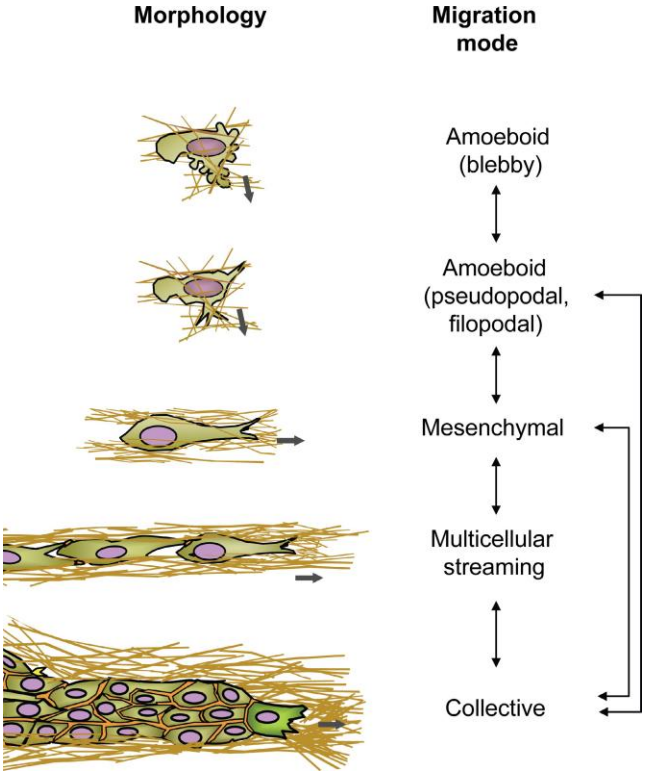

Fig. 2: Cellular modes of interstitial migration. Cells interact with the extracellular matrix (brown). Thick grey arrows indicate direction of movement. Slim black arrows indicate possible transitions between modes of migration. Adopted from Friedl & Wolf, 2010.

chemotherapy than other cancer cells (Gupta *et al.* 2009). The increased survival of cancer stem cells causes difficulty in treatment of the cancer and contributes to an increase of metastases and recurrences (Iwatsuki *et al.* 2010). Due to the stem cellness of mesenchymal cancer cells produced by EMT, mesenchymal cancer stem cells are able to set up metastases and perhaps lead to recurrences (Mani *et al.* 2008; Kurrey *et al.* 2009; Tiwari *et al.* 2012).

EMT can be induced by a plethora of pathways including: TGFβ, PDGF, FGF, IGF, EGF, HGF, TNFα, Hippo, Wnt and Notch signaling combined with hypoxia (Tiwari *et al.* 2012). Most pathways regulate EMT via transcription of the directly involved EMT transcription factors. EMT transcription factors are transcription factors that directly regulate genes involved in EMT. The EMT transcription factors includes the Snail family members Snail1 and -2, the Zeb family members Zeb1 and Zeb2, and Twist1 (Sánchez-Tilló *et al.* 2012). Zeb1 and Zeb2 are zinc-finger-homeodomain proteins. They both bind to the E-box of an E-cadherin gene and repress E-cadherin through different corepressors (Vandewalle *et al.* 2009). Zeb1 and Zeb2 also inhibit the expression of other epithelial proteins which are part of adherens junctions, tight junctions, gap junctions and desmosomes (Vandewalle *et al.* 2009). Zeb1 and Zeb2 are also involved in the activation of mesenchymal genes, such as N-cadherin, vimentin and several metalloproteases (Vandewalle *et al.* 2009). Zeb1 can also inhibit apoptosis and senescence by inhibiting transcription of p15, p21, p63 and p73 (Tiwari *et al.* 2012). Snail family members have a zinc-finger domain that can bind to E-boxes and a SNAG domain which interacts with epigenetic machinery (Sánchez-Tilló *et al.* 2012). Snail1 and Snail2 can inhibit the E-cadherin gene and other epithelial genes, *e.g.* genes involved in adherens and tight junctions (Sánchez-Tilló *et al.* 2012). There is no evidence that Snail1 or -2 regulates N-cadherin expression directly. Snail1 and -2 can inhibit apoptosis, anoikis and senescence by inhibiting p53 and PUMA (Tiwari *et al.* 2012). Basic Helix-Loop-Helix domain transcription factor Twist1 can inhibit the E-cadherin gene through direct binding, or through activation of Snail1 or Snail2. Twist1 can also activate the N-cadherin transcription. Twist1 can inhibit apoptosis though inhibition of p16 and $p19^{ARF}$ (Sánchez-Tilló *et al.* 2012).

EMT enables cancer cells to invade into different tissues. Invading cancer cells can have different modes of migration, for example: amoeboid, mesenchymal, multicellular streaming or collective migration (Fig.

2) (Yilmaz & Christofori 2010; Friedl & Wolf 2010). Amoeboid can be further divided into blebby, pseudopodal and filopodal. These are based on the actin based protrusions the amoeboid cell shows. Though, EMT might enable the first steps of metastasis in carcinomas. During colonization the reverse process of EMT might be important. Mesenchymal to Epithelial Transfer (MET) seems to be needed for colonization in mouse models (Tsai *et al.* 2012; Ocaña *et al.* 2012). In humans, metastasizing mesenchymal cancer cells can undergo MET and re-express E-cadherin in metastases (Chao *et al.* 2010).

EMT enables carcinoma cells to invade into surrounding tissue. While cells are in mesenchymal stem cell mode they are harder to treat, due to their resistance to chemotherapy (Gupta *et al.* 2009; Li *et al.* 2008). If one could inhibit EMT or induce MET in cancer stem cells, this might lead to less invasive cancers and more efficient chemo and radiotherapy. Also the promoting E-cadherin expression or inhibiting N-cadherin might lead to useful anti-cancer therapy. In this thesis focus will be on the E- to N-cadherin switch and review whether regulation of these cadherins is a good target for the inhibition of EMT.

The E- to N-Cadherin switch

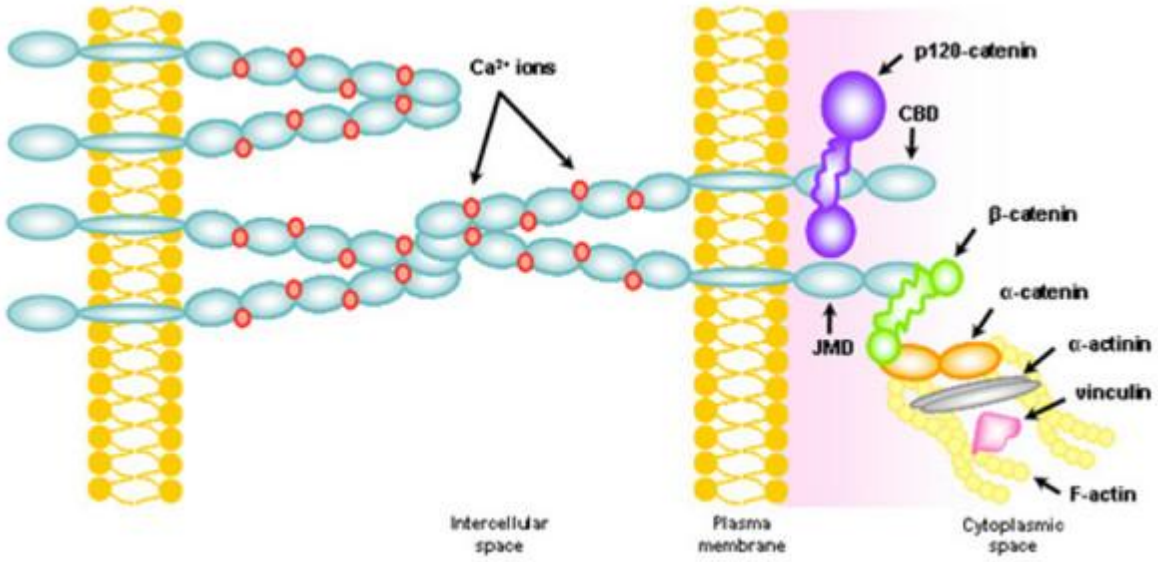

Fig. 3: Classical cadherin cell-cell adhesion. A classical cadherin is a single pass transmembrane protein. Homodimeric cadherins bind through their extracellular EC1 domain. Calcium ions are necessary for cadherin cell-cell adhesion. The intracellular juxtamembrane domain (JMD) of cadherin binds p120-catenin. The catenin-binding domain (CBD) interacts with β-catenin and β-catenin links α-catenin to the cadherin-catenin complex. α-Catenin binds F-actin and α-actinin and vinculin. Adopted from Paredes *et al.* 2007.

During EMT, E-cadherin is suppressed and N-cadherin is upregulated (Kalluri & Weinberg 2009). This is called the E- to N-cadherin switch. E-cadherin guards epithelial integrity and N-cadherin is involved in invasion (Wheelock *et al.* 2008). Cadherins are a family of Ca^{2+} dependent adherens proteins and play a role in development in tissue organization and cell segregation (Leckband & Prakasam 2006). E- and N-cadherin are classical cadherins. Classical cadherins are cell-cell adhesion molecules and have an extracellular domain, single pass transmembrane domain and an intracellular domain (Fig. 3). The E stands for epithelial and the N for neuronal, which is the tissue where these cadherins were first identified (Huveneers & de Rooij 2013). Both E- and N-cadherin localize to adherens junctions. Adherens junctions are cadherin based cell-cell adhesion junction. Adherens junctions with different cadherins might have other functions (Twiss & de Rooij 2013). The extracellular part of cadherin consists of 5 EC domains. The EC domains seem to either homo- or heterodimerize with a cadherin from adjacent cell. However, homodimeric interactions have a higher affinity and only homophilic interactions lead to mechanotransduction (Tabdili *et al.* 2012). The intracellular domain of cadherin bind to $p120^{ctn}$, β-catenin and α-catenin (Twiss & de Rooij 2013)(Derycke & Bracke 2004). Catenin p120 stabilizes adherens junctions and acts as a scaffold protein for Rho GTPases and their targets (Schackmann *et al.* 2013). Catenin p120 has two alternate splice forms in epithelial and mesenchymal cells (Schackmann *et al.* 2013). β-catenin binds to the C-terminal of cadherin and α-catenin can bind to β-catenin (Twiss & de Rooij 2013). β-catenin is a member of the Wnt pathway (Clevers & Nusse 2012) and loss of E-cadherin could lead to more active Wnt pathway, caused by an increase of free β-catenin (Nelson & Nusse 2004).

The increase in Wnt signaling might activate the EMT program (Tiwari *et al.* 2012). α-Catenin in the cadherin/catenin complex acts as a tension sensing signaling complex (Huveneers & de Rooij 2013). α-Catenin does not bind F-actin and β-catenin at the same time, but is thought to play a role in the organization of both (Shih & Yamada 2012a). During EMT, α-Catenin might also be downregulated in some carcinoma's (Benjamin & Nelson 2008).

Table 2: Loss of E-cadherin in carcinoma correlates with lower patient survival

Type of neoplasm	**Loss of E-cadherin correlates with:**		
Prostate carcinoma	Invasion and metastases	(Umbas *et al.* 1994)	
Non-small cell lung cancer	Lymph node metastasis	(Sulzer *et al.* 1998)	
Gastric carcinoma	Increased metastatic potential	(Yonemura *et al.* 2000)	
Vulvar squamous cell carcinoma	Invasive carcinoma	(Zannoni *et al.* 2011)	
Breast cancer	Invasive lobular carcinoma	(Patil *et al.* 2011)	
Colorectal cancer	Differentiation grade, lymph node status and metastasis. Also patient survival in Asian but not European	(He *et al.* 2013)	meta-analysis
Gastric cancer	Invasion, lymph node metastasis, distant metastasis grade of differentiation, vascular invasion and histological type	(Xing *et al.* 2013)	meta-analysis
Lung cancer	Methylation of e-cadherin promoter associated with risk of lung cancer	(Zeng *et al.* 2013)	meta-analysis
Esophageal cancer	Poorer differentiation degree	(Xu *et al.* 2013)	meta-analysis
Ductal breast carcinoma	Invasive carcinoma	(Gould Rothberg & Bracken 2006)	meta-analysis

The expression of E-cadherin in cancer cells is seen as an inhibitor of invasion (Vleminckx *et al.* 1991; Tiwari *et al.* 2012). E-cadherin inhibition is one of the important targets of EMT (Tiwari *et al.* 2012). E-cadherin loss is associated with invasion and metastasis in many carcinomas (Table 2). Knock down of E-cadherin in epithelial cancer cells can induce EMT (Onder *et al.* 2008; Frisch *et al.* 2013; Shimada *et al.* 2012). Although, loss of E-cadherin might not lead to EMT is all epithelial carcinoma cells (Derksen *et al.* 2006; Hollestelle *et al.* 2013). Even though E-cadherin loss might not necessarily lead to EMT, loss of E-cadherin still seems beneficial to tumorigenesis (Derksen *et al.* 2006; Frisch *et al.* 2013). Often E-cadherin loss is accompanied by gain of N-cadherin in carcinomas (Table 3). The gain of N-cadherin leads to an increased invasion rate, both in *in vitro* invasion assays and in *in vivo* models (Derycke & Bracke 2004; Cui & Yamada 2013; Shih & Yamada 2012a). There are exceptions to the E- to N-cadherin switch. First of all, there are also other cadherin switches which are often linked to invasion and metastasis as well (Wheelock *et al.* 2008);(Table 3). For example, other cadherin switches are E- to T-cadherin and E-cadherin to cadherin11. Secondly, E- and N-cadherin are not always mutually exclusive, as in colon carcinomas (Rosivatz *et al.* 2004) and several other endodermal epithelium derived cells (Straub *et al.* 2011). N-cadherin adherens junctions in endodermal epithelial cells are able to interact with

mesenchymal cells (Straub *et al*. 2011). What the exact role of E- and N-cadherin coexpression is unknown. In breast cancer E-cadherin and N-cadherin are not normally coexpressed. Whereas exogenous coexpression of N-cadherin seems to be dominant over the expression of E-cadherin in invasion (Hazan *et al.* 2000)(Hazan *et al.* 2004). The coexpression in some endodermal epithelia indicates N-cadherin expression might have different effects in different tissues.

Table 3: Cadherin switching in tumorigenesis (adopted form Wheelock *et al.* 2008)

Cadherin switch	Type	Associated with	
E to N-cadherin	Melanoma cells	Increased invasion	(Li *et al.* 2001)
	TGFβ dependent EMT in mammary epithelial cells		(Maeda *et al.* 2005)
	Prostate cancer	Prognostic marker	(Gravdal *et al.* 2007; Jaggi *et al.* 2006)
	Breast cancer	Increased invasion	(Hazan *et al.* 2000; Han *et al.* 1999; Nieman *et al.* 1999)
	Pancreatic cancer	Increased metastases	(Hotz *et al.* 2007; Nakajima *et al.* 2004)
E- to T-cadherin	Hepatocellular carcinoma	Increased invasive capacity	(Riou *et al.* 2006)
E- to P-cadherin	Pancreatic cancer	Promotes motility	(Taniuchi *et al.* 2005)
	Gastric cancer		(Shimoyama & Hirohashi 1991)
E-cadherin to cadherin 11	Prostate cancer	Increased invasion	(Bussemakers *et al.* 2000; Tomita *et al.* 2000)
	Breast cancer cells		(Pishvaian *et al.* 1999)
E- and P- to N-cadherin	Oral squamous cell carcinoma	Invasion and metastasis	(Chen *et al.* 2004; Pyo *et al.* 2007)
N- to E-cadherin	Ovarian cancer	Malignant progression	(Patel *et al.* 2003; Wong *et al.* 1999)

Consequences of E-cadherin loss

Loss of E-cadherin function is associated with increased invasion (Vleminckx *et al.* 1991). E-cadherin can be inactivated by promoter hypermethylation by EMT transcription factors (Sánchez-Tilló *et al.* 2012; Christofori 2006; Graff *et al.* 1995), growth factor signaling (Thiery 2002)(Tiwari *et al.* 2012), internalization and degradation (Delva & Kowalczyk 2010), cleavage of the E-cadherin protein (David & Rajasekaran 2012) and loss of protein function through mutations or loss of the exons or the whole genomic region (Berx *et al.* 1998). The mechanistic link between E-cadherin loss and cancer cell invasion has been extensively investigated. Knockdown of E-cadherin can lead to EMT in epithelial breast cancer

cell line HMLE (Onder *et al.* 2008). Onder *et al.* show that knockdown of E-cadherin lead to upregulation of mesenchymal markers N-cadherin, vimentin and fibronectin. In mice, injection of E-cadherin deficient cancer cells lead to the formation of metastases more rapidly than the control (Onder *et al*. 2008). The E-cadherin knockdown cells invade more and are more resistant to anoikis. Furthermore, β-catenin localizes to the nucleus in E-cadherin knock down cells (Onder *et al*. 2008). Both invasion and anoikis resistance were partly dependent on β-catenin. The knockdown of E-cadherin also led to an upregulation of Twist. Invasion and anoikis resistance also partially depended on Twist expression. N-cadherin expression depended partially on Twist, though vimentin expression did not depend on Twist. This indicates that EMT is a multifaceted program. E-cadherin adhesion suppresses EMT. The question arises, what the molecular mechanism of Twist activation after E-cadherin loss is. Twist expression was not dependent on β-catenin (Onder *et al.* 2008). It is possible that Twist expression is dependent on Hippo pathway transcription factors YAP and TAZ. E-cadherin is involved in the Hippo pathway. E-cadherin plays a role in contact inhibition through Hippo signaling (Kim *et al.* 2011). YAP and TAZ are transcription factors of the Hippo pathway. E-cadherin inhibits the activity of YAP by sequestering 14-3-3 through α-catenin binding of 14-3-3 (Schlegelmilch *et al.* 2011). 14-3-3 binds YAP and α-catenin and spatially inhibits YAP from nuclear localization. α-Catenin inhibits phosphatase PP2A from dephosphorylating YAP, releasing the inhibition from 14-3-3. Loss of α-catenin and possibly E-cadherin releases YAP inhibition by 14-3-3. YAP transcriptional activity is involved in regulating EMT (Chan *et al.* 2008). Also, EMT promotes self-renewal through TAZ (Cordenonsi *et al.* 2011). TAZ is one of two transcription factors of the Hippo pathway, the other is YAP. Since Hippo signaling had an important function during EMT, loss of E-cadherin might also induce EMT through the Hippo pathway. This can be researched combining E-cadherin and TAZ knock down in epithelial cancer cell lines. If E-cadherin loss leads to EMT, E-cadherin and TAZ knock down should not lead to EMT.

E-cadherin loss leads to an increased anoikis resistance (Onder *et al.* 2008; Derksen *et al.* 2006). This might partially be due to release of NRAGE. E-cadherin binds to Ankyrin-G and Ankyrin-G sequesters NRAGE (Frisch *et al.* 2013). Loss of E-cadherin leads to release of NRAGE and released NRAGE inhibits the expression of several genes, among which p14ARF (Frisch *et al.* 2013). p14ARF is a inducer of anoikis and apoptosis (Tiwari *et al*. 2012).

Loss of E-cadherin might be important in some cancer cell lines, but there are exceptions. Hollestelle *et al.* studied 38 breast cancer cell lines. 11 were mesenchymal, though 7 had lost E-cadherin protein expression (Hollestelle *et al.* 2013). Furthermore, reexpression of E-cadherin did not reverse the mesenchymal morphology. The authors suggest E-cadherin loss might not be necessary during EMT. They could be right, because the loss of epithelial integrity is important during EMT. Loss of E-cadherin could be a manner to achieve loss of epithelial integrity. Though, loss of E-cadherin might not be a necessity for EMT; perhaps due to presence of other epithelial proteins protecting epithelial integrity. The reason reexpression of E-cadherin does not rescue the mesenchymal phenotype, might be due to other proteins downstream that also could be downregulated. For example, α-catenin could also be downregulated during EMT and is necessary for E-cadherin function (Benjamin & Nelson 2008; Twiss & de Rooij 2013; Hollestelle *et al*. 2013).

In summary, loss of E-cadherin leads to EMT in some cancer cell lines. Though, E-cadherin loss is not necessary for EMT in other cancer cell lines. E-cadherin sequesters β-catenin, 14-3-3 and NRAGE, which might suppress EMT. Release of these factors might lead to EMT in some cell lines.

Conditional mouse models of E-cadherin loss

When E-cadherin is lost in epithelial cancers, cancer cells undergo EMT in mouse models (Shimada *et al.* 2012). Shimada *et al.* describe a p53/E-cadherin conditional knock out mouse model of diffuse type gastric cancer. They tissue specifically knock out p53 and E-cadherin using *Atp4b* promoter driving Cre expression in the parietal stomach lining. Loss of E-cadherin alone is not enough to induce tumors. Double knockout of p53 and E-cadherin lead to invasive gastric carcinoma's in 69% of the double knockout mice. Also mesenchymal markers such as N-cadherin, Twist1, Snail1, vimentin and fibronectin were upregulated in the double knockout mice. Immunohistochemistry showed N-cadherin and Twist1 were highly expressed in invasive parts of primary diffuse gastric carcinoma. This indicates loss of E-cadherin leads to EMT in gastric carcinoma cells. Though, the authors were not able to show effect of p53 knock out, since p53 knock out alone does not lead to gastric cancer in mice. This also indicates p53 and E-cadherin might have synergistic effects in carcinogenesis.

An E-cadherin breast cancer model also uses double knockout mice (Derksen *et al.* 2006; Derksen *et al.* 2011). Derksen *et al.* use E-cadherin and p53 conditional knockout mice are used as model for invasive lobular carcinoma. In 2006 the authors used a *K14* promoter ligated to the Cre gene. *K14* promoter is active in several epithelial tissues, including mammary epithelium. In 2011 the authors used a more specific *Wap* promoter driving Cre expression which only is expressed in mammary epithelium. Similar to the gastric carcinoma model, E-cadherin knockout alone does not induce tumors. Knock out of p53 lead to expansive carcinoma, but usually not invasive. Double knock out of p53 and E-cadherin leads to invasive carcinoma and metastases. Again similar to the gastric carcinoma model, p53 and E-cadherin seem synergistic in carcinoma formation. However, E-cadherin knockout did not lead to EMT in the lobular carcinoma. All primary carcinomas and metastases had epithelial marker CK8 and did not gain mesenchymal marker vimentin. Even though the E-cadherin/p53 knock out carcinomas did not have mesenchymal markers, they did have an increased vascularization and were more resistant to anoikis. Resisting anoikis is a hallmark of EMT (Tiwari *et al.* 2012) and inducing angiogenesis has been linked to EMT transcription factor activity (Sánchez-Tilló *et al.* 2012). This might indicate that the carcinoma cells have undergone partial EMT, which might be possible since EMT seems a multifaceted program (Onder *et al*. 2008). On the other hand, full EMT seems to be transient and only implemented away from the primary tumor. These experiments show E-cadherin knockout is not always enough to induce full EMT, even though E-cadherin loss does increase invasion and anoikis resistance.

In summary, both mouse models show increased invasion and synergy between p53 and E-cadherin. However, only the diffuse gastric carcinoma model shows EMT upon E-cadherin conditional knockout in a p53 conditional knockout background. Perhaps the loss of E-cadherin leads to a partial EMT in the lobular carcinoma model. It is also possible that only in some types of cells E-cadherin loss leads to EMT. Or in the invasive lobular carcinoma model full EMT only occurs outside of the primary carcinoma and therefore is undetectable. There is a possibility that the differences in floxed alleles lead to EMT. In the

diffuse gastric carcinoma model alleles 6 to 10 of E-cadherin gene *CDH1* were floxed. Whereas the invasive lobular carcinoma model alleles 4 to 15 were floxed. It should be investigated whether this difference in floxed alleles could lead to the difference in EMT induction.

E- and N-cadherin, comparison of invasive capacities

The proteins that E- and N-cadherin can interact with are overlapping, *e.g.* p120 catenin, α-catenin and β-catenin (Twiss & de Rooij 2013; Shih & Yamada 2012a). Furthermore, in embryonic mouse heart substituting of N-cadherin for E-cadherin did not affect the development of the heart (Luo *et al.* 2001). Thus, what causes the difference between E- and N-cadherin and their effect on invasion? E-cadherin has a slightly higher affinity for itself, than for N-cadherin. Though, this might not be enough to explain the difference in invasion (Tabdili *et al.* 2012). In 3D matrices the invasion of N-cadherin positive cells occurs in train like formation and N-cadherin localizes to adherens junctions between the invading cells (Shih & Yamada 2012b). Knockdown of N-cadherin lead to round up single cells that do not invade (Shih & Yamada 2012b). This might indicate that N-cadherin adherens junctions are necessary for invasion in a 3D matrix. The difference between the effects of N- and E-cadherin on cell behavior might be caused by the interaction with α-catenin. Both E- and N-cadherin can interact with α-catenin. In PC9 cells, the expression of α-catenin in N-cadherin positive and E-cadherin negative cells lead to an increase of cell-cell adhesion and a decrease of invasion in a 3D matrix (Cui & Yamada 2013). This suggests that α-catenin can increase N-cadherin adhesive qualities. The presence of α-catenin might stabilize N-cadherin – N-cadherin interactions, though this has to be investigated. The inactivation α-catenin during might be very important during EMT.

Another mechanism by which N-cadherin could promote invasion is via association with growth factor receptors Fibroblast Growth Factor Receptor-1 (FGFR-1) and possibly Platelet Derived Growth Factor Receptor β (PDGF-Rβ). Association of N-cadherin with the PDGF-Rβ receptor increases *in vitro* wound healing capabilities (Theisen *et al.* 2007), though an effect on invasion has not been tested. Additionally, N-cadherin can inhibit Akt3 in breast cancer, leading to an increase of invasion (Chung *et al.* 2013). Furthermore, the association of N-cadherin with FGFR-1 increases the activity of FGFR-1 (Suyama *et al.* 2002). The increased FGFR-1 activity leads to an attenuated ERK and AKT1/2 pathway promoting EMT via Snail1 and Snail2 upregulation (Qian *et al.* 2013). The contribution of N-cadherin to FGFR-1 activity drives proliferation, invasion and stem cellness. All these together increase the chance of a cancer cell metastasizing successfully. N-cadherin expression seems to be dominant over E-cadherin expression (Hazan *et al.* 2004). The reason N-cadherin expression is dominant over E-cadherin expression might be FGFR-1 activation. Using chimera of E- and N-cadherin it was found that the EC4 domain of N-cadherin alone decreased cell-cell adhesion (Kim *et al.* 2000). Furthermore, N-cadherin with the EC4 of E-cadherin increased cell-cell adhesion. This might indicate EC4 is an essential domain in determining the invasive capabilities of E- and N-cadherins. EC4 is also the domain of N-cadherin found to interact with FGFR-1 (Suyama *et al.* 2002). Targeting this domain with an antibody impaired N-cadherin depend invasion (Kim *et al.* 2000).

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Furthermore, cadherins can regulate the cytoskeleton by modulating the activity of small GTPases (Watanabe *et al*. 2009). E-cadherin activates small GTPases RhoA, CDC42 and Rac1. Whereas N-cadherin activates RhoA and inhibits CDC42 and Rac1 in myoblasts (Charrasse et al. 2002; Watanabe *et al*. 2009). N-cadherin localized at cell-cell junctions inhibited Rac1 and PI3K (Ouyang *et al.* 2013) and excluded integrins from the cell-cell junctions (Ouyang et al. 2013). Also N-cadherin suppresses membrane protrusions which aids in migration (Cui & Yamada 2013). Differences in modulation by cadherins of small GTPases and possibly integrins might very well lead to observed differences in migration and invasion.

In conclusion, the main differences between the N- and E-cadherin seem to be in interactions with other proteins, not as much differences in affinity between homodimeric complexes.

Targeting EMT

Low dosage chemotherapy with paclitaxel inhibits EMT *in vitro*, and perhaps do so *in vivo* as well (Hirose *et al.* 2013). Thus there is a possibility that paclitaxel also inhibits EMT in carcinomas. The inhibition of EMT might be a useful target for anticancer therapy and more drugs targeting EMT are being developed. The rationale for suppressing EMT in cancer is to inhibit the formation of new cancer stem cells and metastases. Locking cancer cells in epithelial state might also be accomplished by drugs inducing MET. Locking cancer cells in epithelial state and using chemotherapy might prove useful. Since, it might lead to a smaller group of cancer stem cells, making the tumor more susceptible to chemotherapy *(Gupta et al*. 2009). The development of drugs often starts with screening compound libraries for an effect on cells. By screening mesenchymal cancer cells for re-expression of E-cadherin protein one might be able to identify drugs capable of inhibiting invasion (Hirano *et al.* 2013). Hirano *et al.* show two compounds are capable of re-expressing E-cadherin. These compounds inhibit of invasion in 3D invasion assays in which the cells treated with the compound only invaded 1/5 compared to control. One of those compounds inhibited the ERK and AKT pathways. Activation of the AKT1 pathway is increases Twist expression (Khan *et al.* 2013). It is possible that these compounds induce MET. To investigate this, staining the compound treated cells for other epithelial and mesenchymal markers might be an option.

Another method of screening drugs for expression of epithelial and mesenchymal markers is by screening compounds for an effect on cell morphology. Or by screening for drugs capable of inhibiting cell lines to go through EMT in response to an EMT inducing ligand (Chua *et al.* 2012; Reka *et al.* 2011; Loerke *et al.* 2012). By using software that assesses characteristics of EMT, such as cell scattering and morphology, you can screen vast amounts of compounds in shorter time. However you only screen drugs inhibiting the inducer of the EMT, for example inhibitors of HGF pathway if HGF is used as EMT inducer. Further characterization of hits is needed, such as effects of the drug on invasive capabilities of cancer cells and cell viability. To identify which compounds are useful in which genetic backgrounds, one can use an *in vivo* RNAi screen. By using an *in vivo* RNAi screen on a model undergoing EMT, such as the p53/E-cadherin conditional knockout diffuse gastric carcinoma model (Shimada *et al.* 2012), one could identify useful target genes. By isolating invasive cancer cells, transfecting them with a shRNA library you can assess which RNAi targets are helping invasion and which are inhibiting invasion. By comparing the depletion of shRNAs in invading cancer cells or metastases, you can identify new drug targets (Gargiulo *et al.* 2013). By combining these data you can identify a compound that inhibits a protein and thereby inhibits EMT and be relatively sure that targeting that protein inhibits invasion in that background. This knowledge can be translated to the clinic and used in personal medicine.

Chemotherapy does not target cancer stem cells very efficiently. Therefore, a screen has been set up to target mesenchymal cancer stem cells (Gupta *et al.* 2009). Gupta *et al.* knocked down E-cadherin induce EMT and create cancer stem cells in the HMLE breast cancer cell line. They identified several compounds that kill cancer stem cells, but not normal cancer cells. In the future these compounds might be used in combination with chemo-, radiotherapy and surgery. However, the question remains whether the drugs kill normal stem cells as well. The E-cadherin knockdown cell line inducing EMT could be used to identify compounds that induce MET in absence of E-cadherin.

Another way to target mesenchymal cancer stem cells is through use of antibodies. Antibodies targeting mesenchymal markers might target cancer stem cells and inhibit metastasis. N-cadherin drives invasion (Cui & Yamada 2013; Shih & Yamada 2012b). N-cadherin antibodies can be used to suppress growth and metastasis in prostate cancer (Tanaka *et al.* 2010). N-cadherin positive cells had stem cell characteristics and addition of the N-cadherin antibody 2A9 lead to apoptosis of neoplastic tissue at some dosages. Antibody 2A9 targets the fourth extracellular EC domain of N-cadherin. This domain is known to associate with FGFR-1 (Suyama *et al.* 2002). Antibody 2A9 reduces AKT phosphorylation, though the authors did test the AKT homologues separately. Since N-cadherin and FGFR-1 dimerization promotes AKT2 phosphorylation (Qian *et al.* 2013), 2A9 might inhibit dimerization of N-cadherin and FGFR-1. The authors did not test this assumption. They might test FGFR-1 phosphorylation in presence and absence of antibody 2A9. This might indicate attenuation of the FGFR by antibody 2A9. The next step would be to test if an effect on FGFR by 2A9 is dependent on N-cadherin. This antibody might also be useful for targeting mesenchymal cells in other cancers were N-cadherin is upregulated. N-cadherin expression is not limited to cancer stem cells. Therefore, side effects might be expected, yet, the authors do not report side effects.

In conclusion, development of anticancer drugs inhibiting EMT of promoting MET is still in test phase. With more research, drugs useful in clinic might be identified. Described papers have at least shown that EMT can be suppressed and MET can be promoted with use of compounds. The use of such drugs could lead to more chemo sensitive cancer cells, which are less invasive. Perhaps EMT suppressing drugs could be combined with drugs targeting cancer stem cells (Chen *et al.* 2013) and chemo targeting the bulk of the tumor.

Discussion and Conclusion

In carcinomas, the EMT process can turn epithelial cancer cells into stem cell mesenchymal cancer cells (Mani *et al.* 2008; Gupta *et al.* 2009). Mesenchymal cells might be able to set up metastases and are considered harder to target by chemotherapy (Gupta *et al.* 2009). The E- and N-cadherin switch can be a part of EMT in some carcinomas, *e.g.* melanoma, prostate, breast and pancreatic carcinomas (Table 3). Although, there are also other types of cadherin switches in carcinomas, sometimes also linked to invasion. E-cadherin is involved in cell-cell adhesion while epithelial integrity and N-cadherin can regulate cell-cell adhesions during invasion (Tiwari *et al.* 2012; Shih & Yamada 2012a).

In mouse models of carcinoma, E-cadherin knockdown leads to invasive carcinomas (Derksen *et al.* 2006; Derksen *et al.* 2011; Shimada *et al.* 2012). In a diffuse gastric carcinoma model, E-cadherin and p53 loss leads to invasive carcinomas with mesenchymal characteristics (Shimada *et al.* 2012). In the invasive lobular carcinoma mouse model, loss of E-cadherin and p53 leads to invasive carcinomas, however, there was no evidence indicating that carcinoma cells underwent EMT (Derksen *et al.* 2006; Derksen *et al.* 2011). Though, Derksen *et al.* only tested vimentin and smooth muscle actin, thus expression of other mesenchymal markers could be tested. The lack of EMT raises the question of how well the invasive lobular carcinoma model compares to human invasive lobular carcinoma, since in human carcinoma EMT was found to play a role in carcinoma progression (Aigner *et al.* 2007). It is possible that only invading cancer cells in the invasive lobular carcinoma mouse model underwent a temporal EMT which was not present in the primary carcinomas and metastases. In that case, it might possible to show that EMT is presence or absence of mesenchymal cancer cells in the blood circulation (Yu *et al.* 2013). E-cadherin loss alone does not lead to carcinogenesis in either mouse model. Neither did knockout of p53 in the gastric carcinomas model (Shimada *et al*. 2012). Knockout of p53 does lead to lobular carcinomas, although they are not invasive (Derksen *et al.* 2006). In both the gastric and lobular carcinoma mouse models E-cadherin and p53 loss was found to act synergistically in carcinogenesis. The cause of synergy in p53 and e-cadherin loss in carcinoma formation is not fully understood. It is possible that loss of p53 and E-cadherin are both steps on the ladder to invasive carcinoma. Knockdown of p53 leads to inhibition of apoptosis and E-cadherin loss to a decreased epithelial integrity and increased invasion. These are both hallmarks of carcinogenesis (Hanahan & Weinberg 2011). Other mutations might further enable carcinogenesis, *e.g.* mutations leading to sustained proliferation and a limitless replicative potential. The loss of E-cadherin might lead full or partial mesenchymal state via the activation of Wnt and Hippo pathway transcription factors (Nelson & Nusse 2004; Kim *et al.* 2011). Both the Wnt and Hippo transcription factors are β-catenin and YAP/TAZ respectively. YAP/TAZ and β-catenin transcriptional activity inhibits anoikis (Onder *et al.* 2008; Kim *et al.* 2011; Zhao *et al.* 2012). The loss of E-cadherin also leads to the release of NRAGE. NRAGE is associated with Ankyrin-G and Ankyrin-G is bound to E-cadherin in the cell membrane. Release of NRAGE leads to inhibition of p14ARF transcription and anoikis, because p14ARF is a promoter of anoikis (Frisch *et al.* 2013). Furthermore, the increased activity of transcription factors β-catenin and YAP/TAZ might lead to increased proliferation and stem cellness (Cordenonsi *et al.* 2011; Schlegelmilch *et al.* 2011; Holland *et al.* 2013). The activated Wnt and Hippo transcription factors could be able to induce EMT, perhaps via upregulation of Twist or other EMT transcription factors (Tiwari et al. 2012; Onder *et al.* 2008). This might enable further carcinogenesis through inhibition of apoptosis, dedifferentiation and inhibiting cellular senescence. Crosstalk between

transcription factors and EMT transcription factors might enhance EMT. For example, the ERK1/2 dependent interaction between snail1 and β-catenin induces EMT (Zucchini-Pascal *et al.* 2013). The combined loss of p53 and E-cadherin might lead to inhibition very strong inhibition of apoptosis and anoikis. Genomic instability which would normally lead to apoptosis now has a free hand, leading to more mutations. Genomic instability is probably necessary for tumorigenesis, because not all E-cadherin/p53 knock out cells lead to tumors. Thus which mutations are necessary for further tumorigenesis in E-cadherin/p53 knockout models? This could be tested using in vivo RNAi in E-cadherin/p53 conditional knockout mice. Another way might be to sequence the tumors of those mice for shared mutations. Loss of E-cadherin alone probably does not inhibit anoikis strongly enough and genomic instability still leads to p53 activation paired with senescence and apoptosis (Zilfou & Lowe 2009). Whether inhibition of anoikis is dependent in E-cadherin/p53 conditional knockout mice on YAP, β-catenin and NRAGE can be examined is isolated carcinoma cell lines of the invasive lobular carcinoma model. By making combinations of knockdown of YAP/TAZ, β-catenin or NRAGE one can investigate whether these affect anoikis. How the invasive lobular breast cancer model compares to human cancer

In summary, the loss of E-cadherin is a step in the transformation into mesenchymal cells. However, how big that step is might be tissue and cell line specific. In some cancer lines the knockdown of E-cadherin might lead to EMT due to other mutations. In mice models of invasive carcinomas EMT has been observed following knockdown of E-cadherin and p53 (Shimada *et al.* 2012). However, carcinomas are not invasive immediately and might need to acquire extra mutations to lose epithelial integrity and start invading. What other mutations are necessary to induce EMT besides E-cadherin might be investigated using RNAi in an epithelial cell line. Knock down of E-cadherin alone might not lead to EMT in a non-cancer cell line. By knocking down other genes in E-cadherin knock down cells, other EMT suppressing genes might be discovered

Comparing E- and N-cadherin

Where E-cadherin expression is often lost during EMT, N-cadherin is regularly upregulated (Tiwari *et al.* 2012). N-cadherin expression drives invasion by regulating cell-cell adhesion in multicellular streaming in 3D substrates (Shih & Yamada 2012b; Cui & Yamada 2013; Shih & Yamada 2012a). N-cadherin suppresses cellular protrusions of follower cells, which could keep the cells in a group while invading. Invasion is likely also aided by downregulation proteins involved in cell adhesion such as α-catenin (Cui & Yamada 2013). N-cadherin also associates with growth factor receptors such as FGFR-1 and PDGF-Rβ (Qian *et al.* 2013; Theisen *et al.* 2007). The association with FGFR-1 increases the activity of FGFR-1, leading to an increased invasion and a stem cell signature. N-cadherin and E-cadherin also have differential effect on small Rho GTPases (Watanabe *et al.* 2009; Charrasse *et al.* 2002). The differences in interaction partners might be the reason why E-cadherin suppresses invasion and N-cadherin increases invasion. Especially the interaction with FGFR-1 and N-cadherin might drive invasion, since a chimera of E-cadherin with the FGFR-1 interacting domain EC4 of N-cadherin also decreased adhesion. While an N-cadherin with EC4 of E-cadherin increased cell-cell adhesion (Kim *et al.* 2000). EC4 is the domain of N-cadherin that interacts with FGFR-1. To test the assumption that EC4 of N-cadherin in E-cadherin associates with FGFR-1, one might do a coimmunoprecipitation experiment to measure the interaction between E-cadherin chimera with an N-cadherin EC4 domain and FGFR-1.

Targeting EMT

In various settings, extensive screens have been performed to identify compounds inhibiting EMT or inducing MET (Chua *et al.* 2012; Reka *et al.* 2011; Loerke *et al.* 2012). Some are testing compounds for inhibition of an EMT inducing pathway; others are testing for reexression of epithelial markers such as E-cadherin. Even though re-expression of E-cadherin alone might not be enough to induce MET (Hollestelle *et al*. 2013). Therefore, other epithelial adhesion proteins are likely also upregulated. Antibodies targeting mesenchymal markers such as N-cadherin can also inhibit metastasis (Tanaka *et al.* 2010). This antibody also seems to target cancer stem cells in prostate cancer. Chemotherapy does not target cancer stem cells very efficiently (Gupta *et al.* 2009). By suppressing EMT or inducing MET, one might lock cancer cells in epithelial state. This would decrease the formation of new cancer stem cells and metastases. Furthermore, locking cells in epithelial state might increase the efficiency of chemotherapy. In the future, conventional anticancer therapy might be combined with drugs targeting cancer stem cells (Gupta *et al.* 2009). This could also be combined with drugs targeting cancer cells specifically, some already in clinic such as Herceptin. Herceptin is an monoclonal antibody targeting the Human Epidermal Growth Factor Receptor 2 (HER2) (Baselga *et al.* 1998).

In conclusion, EMT is an important step in metastasis and can supplement the cancer stem cell population. Both are negative processes to patient survival. If you could successfully target EMT in cancer cells this might aid greatly in the patient survival by combining these drugs with chemotherapy. If you could target the cancer stem cells at the same time, this might diminish the chance of metastases and recurrence. Side effects are hard to predict, since our understanding of EMT in both healthy and diseased tissue is still limited and pathways targeted also play a role in other processes, such as wound healing (Kalluri & Weinberg 2009).

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