From leaving the Old World to colonizing the New World:

A role of epithelial-mesenchymal transitions in carcinogenic progression

"By prevailing over all obstacles and distractions,
one may unfailingly arrive at his chosen goal or destination."

Kay Wiebrands
November 2010

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The epithelial-mesenchymal transition plays an important role in several developmental processes, tissue repair, but is also associated with fibrosis and cancer. During tumorigenic progression, EMT pathways are used by cancer cells to gain a migratory and invasive phenotype, enabling these cells to metastasize. This increased migratory potential relies on major changes in, for example, cell adhesion molecule (CAM) expression and cytoskeletal reorganization. In this thesis, I discuss the most important transcription factors during EMT, the effects these genes exert at the cellular level, and how these transcription factor aid carcinogenic progression during the different steps of metastasis. Studying the changing cell states during EMT and the responsible signaling pathways during these processes will enhance our understanding of the metastatic cascade and lead to better targeted therapies.

Introduction

"Omnis cellula e cellula", Rudolf Virchow stated in 1863 in his book 'Cellular Pathology'. Virchow postulated his controversial idea that every cell arises from another cell while he was studying cancer and was subsequently seen as founder of cellular pathology. Starting with the zygote, proliferation is required to form all tissues and organs in the adult body. Furthermore, as can be seen by the ultimate diversity of tissues in a mature organism, cells can assume different fates. This process, known as differentiation, provides cells with their distinct identities and specialized functions. Initially, it was thought that differentiation is a one-way process, and that terminally differentiated cells, i.e. cells of the epithelium, are unable to lose their individuality and differentiate towards a cell type of another lineage. However, more recent studies have shown that cells of the epithelium can actually dedifferentiate towards a mesenchymal state, enabling the cells to migrate and form new structures at a distant site. This epithelial-mesenchymal transition (EMT) is a crucial biological process during embryonic development and wound healing. Next to these physiological events, EMT pathways are also activated during pathologies. Cancer cells can go through EMT to detach from the primary tumor, migrate towards the vasculature, get transported through the body by the blood circulation, and settle at a distant site where metastatic foci can be formed.

This thesis provides a short overview on the role of EMT during development and wound healing, but is focused mainly on EMT during the different steps of the metastatic cascade. I will discuss the key transcription factors associated with cancer progression and the effects of these factors on the expression of different cell adhesion molecules (CAMs) and the cytoskeleton, which are key molecules in this process.

EMT in development and tissue regeneration

EMT involves the formation of a motile mesenchymal cell from an immobile epithelial predecessor (Figure 1). In literature three different subtypes of EMT are recognized. Although EMT per definition results in the formation of migratory mesenchymal cells, the three subtypes all represent a specific biological event (Zeisberg and Neilson, 2009). The earliest EMT (type 1 EMT) in mammals occurs even before implantation during embryonic development. Then after implantation, EMT is required to start gastrulation, which results in the formation of the three germ layers and proper positioning of the cells in relation to the body plan (Wolpert, 2002). During type 1 EMT, primary epithelial cells of the embryo, derived from the ectodermal germ layer, give rise to primary mesenchymal cells that subsequently detach from each other and the basement membrane to ingress through the primitive streak. The migrating mesenchymal cells either remain mesenchymal and form the mesoderm, or undergo the reverse process, mesenchymal-epithelial transition (MET), and give rise to the third germ layer, the endoderm (Figure 2A) (Thiery et al., 2009). Thus, EMT is not irreversible and MET, like EMT, is also observed during embryonic development. Multiple rounds of switches between the epithelial and mesenchymal phenotype are required for appropriate differentiation and localization of cells during the formation of a mature organism (Thiery et al., 2009). After gastrulation is finished, EMT remains crucial during development of the different organs. More examples of type 1 EMT include formation of the neural crest cells, the cardiac valves, skeletal muscle, endocrine cells of the pancreas and

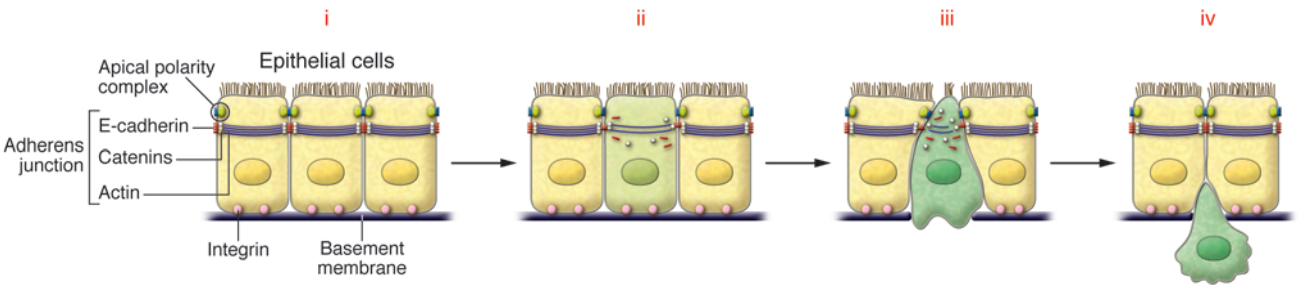

Figure 1 | EMT. In epithelial cells, E-cadherin and integrins provide cell-cell and cell-basement adhesion, respectively (i). During EMT, the transcription of E-cadherin and integrins in epithelial cells is repressed, inducing the loss of polarity in these cells (ii). To invade the underlying tissue, the basement membrane is disrupted and the cytoskeleton remodeled to induce a migratory morphology (iii). This allows the cells to detach from the epithelial sheet and move into the surrounding stroma (iv) (Acloque et al., 2009).

hepatoblasts of the liver (Thiery et al., 2009). In conclusion, type 1 EMT generates cellular plasticity, in order to form new structures and tissues in the developing embryo.

Besides playing a role in developmental processes, EMT is also observed in mature organisms. Upon injury, keratinocytes at the leading edges of the wound undergo dramatic phenotypic alterations, including altered cell adhesion molecule expression, cytoskeletal rearrangements and enhanced migratory characteristics (Figure 2B). Taken together, these keratinocytes undergo EMT-like processes, enabling them to migrate and re-colonize the wound with a new layer of epithelium (Arnoux et al., 2008). Next to wound healing, type 2 EMT is also involved in tissue regeneration. During liver injury, for example, EMT is one of the mechanisms promoting repair. Liver cells that have undergone EMT and repopulate the tissue subsequently revert back to hepatocytes by MET (Choi and Diehl, 2009). However, unlike type 1 EMT, fibrosis can also be an outcome of type 2 EMT, and so a physiological response to injury can result in a pathological event. Type 2 EMT involves the transition of an epithelial cell to a fibroblast, generated to reconstitute the damaged tissue. Type 2 EMT is closely linked to inflammation, but normally inflammation ceases and homeostasis is regained after a wound is healed. However, in the context of fibrosis the inflammation persists and fibroblastic cells accumulate in the tissue, secreting large concentrations of collagen. This excessive collagen deposition then inhibits organ function and can ultimately lead to organ failure and even organ destruction (Thiery et al., 2009).

EMT during cancer progression and metastasis

Next to the possible pathological manifestations of type 2 EMT, type 3 EMT is associated with cancer progression and occurs in epithelial tumors, which account for 90% of the human tumors (Kalluri and Weinberg, 2009; Klymkowsky and Savagner, 2009). As a result of the genetic and epigenetic alterations that occur in a progressing tumor, cancer cells acquire certain capabilities that distinguish them from regular epithelial cells. These capabilities include increased replicative potential, reduced apoptosis and induction of angiogenesis (Hanahan and Weinberg, 2000). However, the capability of cancer cells to detach from the tumor, invade the tissue, enter the circulation and start a distant colony is what makes cancer difficult to treat. Cancer cells that obtain this invasive phenotype are, according to many reports, generated by activated type 3 EMT pathways (Figure 2C) (Thiery, 2002). Typically, tumor cells at the invasive front of the primary tumor lose expression of cell adhesion molecules, dissociate from their neighboring epithelial cells and become single motile cells entering the metastatic process, a process resembling EMT (Brabletz et al., 2001). Other data from solid epithelial tumors and various *in vitro* cancer cell lines verify that these cells indeed undergo partial or complete EMT (Thiery, 2002).

At the molecular level, transcription factors that play key roles during all types of EMT are Snail and Twist. By repressing E-cadherin, the major calcium-dependant cell adhesion molecule (CAM), these two factors disrupt the epithelial junctions. In fact, functional loss of E-cadherin is a critical step of EMT, and is considered to be one of the hallmarks of the transition (Peinado et al., 2007). Furthermore, next to cellular adhesion, Snail and Twist also play important roles in remodeling the cytoskeleton, inducing cellular movement and promoting EMT in other ways (Thiery et al., 2009). Not surprisingly, the expression of Snail and Twist have shown to be significantly correlated with cellular survival, proliferation and tumor relapse in several types of carcinoma, indicating that the

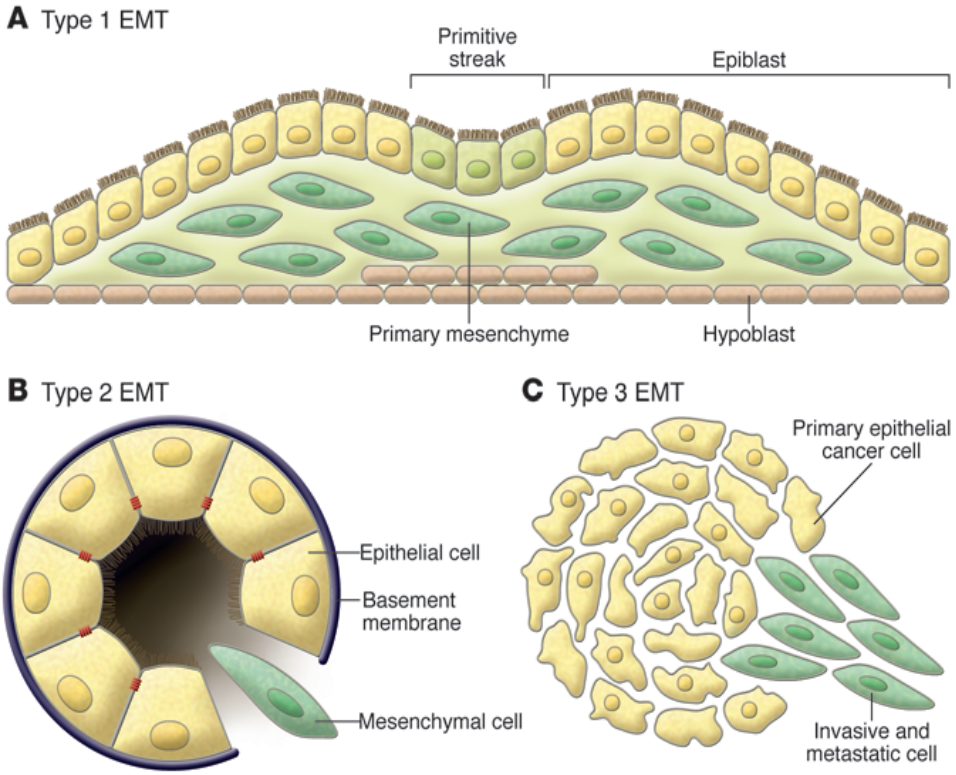

Figure 2 | Three different types of EMT in development and disease. (**A**) Type 1 EMT is associated with embryonic development and plays an important role during implantation and gastrulation, forming the germ layers. Primary epithelial cells use EMT to give rise to primary mesenchymal cells, which subsequently migrate into the underlying tissue through the primitive streak. The motile mesenchymal cells can either remain in their new state and form the mesoderm, or form the endoderm via MET. (**B**) Physiological type 2 EMT is required for wound healing and tissue regeneration. However, when the EMT-inducing stimulus , i.e. inflammation, does not subside over time, organ fibrosis can result in organ failure. (**C**) Cancer cells can also hijack cellular EMT processes. This type 3 EMTs provides epithelial cancer cells with a migratory phenotype, resulting in tissue invasion and metastasis (Kalluri and Weinberg, 2009).

occurrence of EMT leads to poor clinical outcome (Thiery et al., 2009).

Although it may seem obvious that epithelial cancer cells, like the normal cells they derive from, go through EMT to lose their adhesive phenotype and become migratory, the significance of EMT in tumor progression remains debated and several aspects of the contribution of EMT to the metastatic phenotype require additional investigation (Tarin et al., 2005). First of all, although a common set of genes and biological factors are at the base of all types of EMT, the specific signals inducing EMT in cancer cells appear to differ from the pathways involved in type 1 and 2 EMT (Kalluri and Weinberg, 2009). The set of mesenchymal markers found on carcinoma cells undergoing EMT is subsequently more diverse than that found on cells during other EMTs, and type 3 EMT is even linked to the gain of epithelial stem cell properties (Mani et al., 2008). This deviation from the other types of EMT, amongst other reasons, is why the role of EMT in cancer metastasis is still under debate as being essential for the initiation of the metastatic process (Tarin et al., 2005). Furthermore, the epithelial markers used in the clinic to identify a mesenchymal cell that has undergone full EMT should be lost if EMT occurs. Instead, a post-EMT cell would be generated, expressing a set of mesenchymal markers identical to the mesenchymal markers present on non-EMT stromal cells (Klymkowsky and Savagner, 2009). Lastly, because EMT is a dynamic and reversible process, post-EMT cancer cells are hard to identify *in vivo*. The fact that distant metastases, that presumably have undergone EMT, are phenotypically very similar to the primary tumor cells from which they originated, is another paradox in the EMT theory. Upon completing the migratory phase of the metastatic cascade and before colonization, the cells must have entered a MET-like program, regaining their original epithelial and proliferative characteristics (Brabletz et al., 2001; Kalluri and Weinberg, 2009). Recent advances in intravital imaging techniques, with the power to follow the migration of orthotopic tumor cells in their endogenous microenvironment, will provide insight into cancer cell behavior *in vivo* and will illuminate the full metastatic process, including the role of EMT and MET herein (Condeelis and Segall, 2003). In the Discussion section of this thesis intravital imaging and other techniques that could enlighten the exact function of EMT during cancer progression will be discussed in more detail.

The next chapters will focus on the most important transcription factors driving EMT, and how they influence CAM expression, cytoskeletal

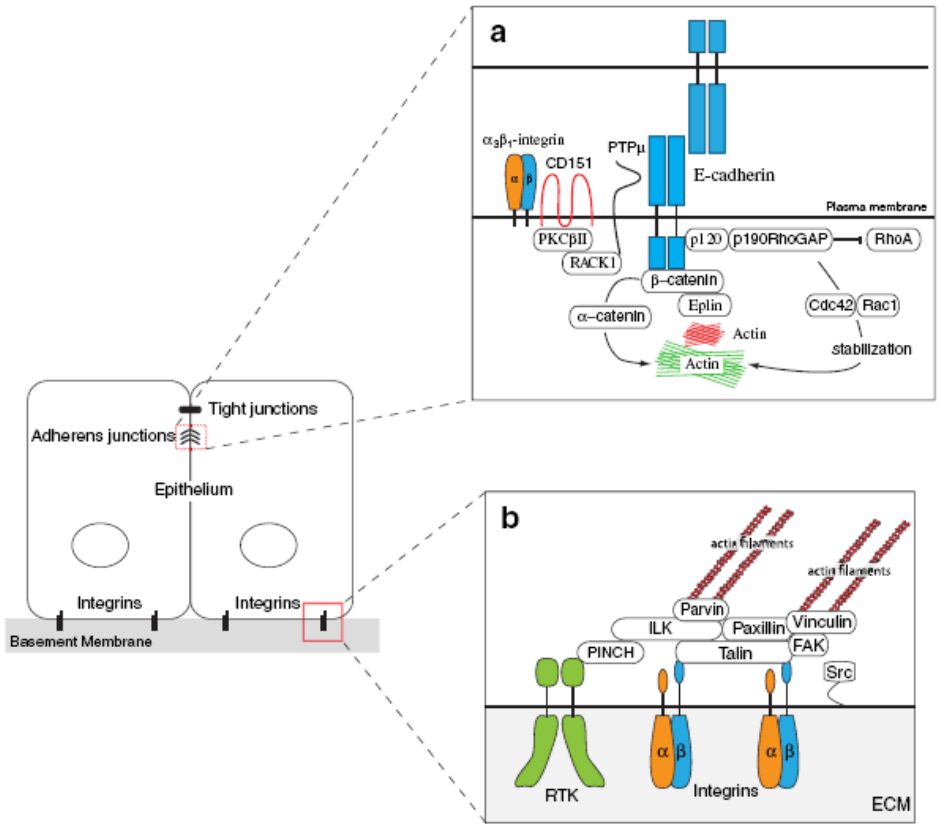

Figure 3 | Cell-cell and cell-matrix interactions of epithelial cells. (A) E-cadherin mediates the main cell-cell interaction in epithelial cells, forming homodimeric bonds with E-cadherin molecules present on neighboring cells. The interactions are strengthened by from the cytoplasmic connections to the actin cytoskeleton, linked to E-cadherin through β- and α-catenin. A variety of other proteins help stabilizing the junctions intracellularly. **(B)** Cells are connected to the ECM by integrins. This family of adhesion proteins consists of an α and β subunit and is coupled to actin filaments by a multiprotein adaptor complex referred to as the IPP complex (Yilmaz and Christofori, 2009).

reorganization, and other events important in EMT. Each chapter will concentrate on a different step of metastasis: detachment of the cancer cells from the primary solid tumor (1) and invasion of the cells into the underlying stroma (2), followed by intravasation into a blood vessel or ducts of the lymphatic system (3), and finally extravasation and colonization of the disseminated cancer cells at a secondary site in the organism (4).

1 Detachment

The cascade of events an epithelial cell has to go through in order to become metastatic and colonize a distant site can be described by several consecutive steps. First, as described above, a cell has to lose contact with its neighboring cells, since epithelial cells are tightly connected to each other to maintain a physical barrier between the internal and external environment. This barrier protects the host from pathogenic invaders, chemical and physical assaults from the external world, while maintaining homeostasis by preventing the loss of water and salts (Proksch et al., 2008). Because of these strong intercellular junctions, remodeling of cell adhesion molecules is required as a first step in EMT. Furthermore, when a cell has lost contact with its neighboring cells, reorganization of the cytoskeleton is necessary to change the cellular morphology and to become migratory (Figure 1). To establish these changes, several intracellular signaling pathways are activated, involving multiple transcriptional regulators. In this chapter, I will discuss important transcription factors and the effects of these factors on the extracellular repertoire of cell adhesion molecules and the cytoskeleton during detachment.

To complete the first step of EMT, detachment from the epithelial sheet, cells first have to downregulate E-cadherin, the main cell adhesion molecule of epithelia. As a member of the cadherin family, E-cadherin interacts with E-cadherin molecules present on the surface of neighboring cells in a calcium-dependent manner. The E-cadherin complexes are intracellularly anchored to actin filaments of the cytoskeleton by their cytoplasmic part, through interaction with β-catenin (Figure 3A). β-catenin can subsequently bind α-catenin, which forms a connection with actin (Kemler, 1993). Thus, via this association with actin, E-cadherin is also involved in remodeling the

cytoskeleton, a role that will be discussed in the next chapter.

As stated above, loss of E-cadherin-mediated cell adhesion leads to a migratory phenotype and coincides with the transition of a benign adenoma to an invasive carcinoma (Perl et al., 1998). Which cellular mechanisms regulate this loss of E-cadherin as a first step towards becoming a metastatic cancer cell? In the literature, a classification is made based on the effects that repressor proteins have on the E-cadherin promoter. One group of transcription factors bind and directly repress the promoter of E-cadherin, whereas the second group represses the transcription of E-cadherin indirectly (Thiery et al., 2009). The earlier mentioned Snail factors belong to the first group of EMT inducers, while the other key factor mentioned in the introduction, Twist, is an indirect E-cadherin repressor.

Snail1 and Snail2 (formerly known as Slug), both members of the Snail family of zinc finger proteins, are associated with the induction of EMT. The involvement of the Snail proteins during all three different types of EMT indicates the importance of these factors during the transition, reaching beyond the regulation of E-cadherin expression alone (Barrallo-Gimeno and Nieto, 2005). In relation to tumor progression, Snail1 expression is detected in various invasive carcinomas and coupled to metastasis (Peinado et al., 2007), as could be expected from a repressor of E-cadherin. Furthermore, both Snail1 and Snail2 are associated with poor clinical outcome (Peinado et al., 2007).

Although the Snail family is the best studied group of direct repressors of E-cadherin, other factors also play significant roles in the inhibition of E-cadherin expression. ZEB1 and ZEB2, for example, two proteins of the ZEB family of zinc finger proteins, can also directly bind to the E-cadherin promoter, repress its expression and induce EMT (Peinado et al., 2007). Like the Snail factors, both members of the ZEB family have also been implicated in malignant progression of human tumors (Peinado et al., 2007).

Another group of E-cadherin repressors, the basic helix-loop-helix (bHLH) factors, including Twist, have been found to be important during invasion and metastasis. bHLH factors homodimerize or heterodimerize to function as repressors and either directly or indirectly inhibit transcription of their target genes. Two members of this family, E47 and Twist, are repressors of E-cadherin, albeit that E47 binds to the promoter directly, while Twist does not directly block transcription (Yang and Weinberg, 2008). Next to these most important and best studied factors, the

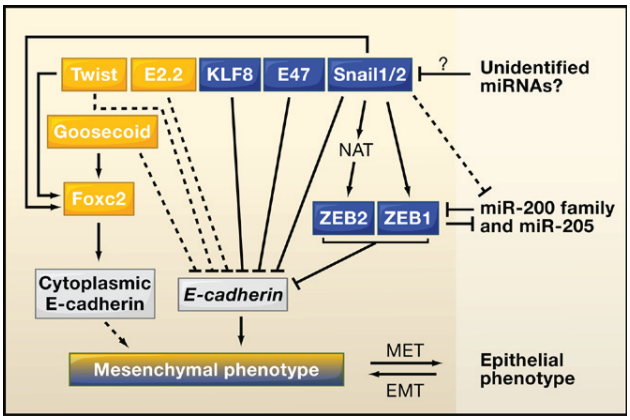

Figure 4 | Transcriptional repression of E-cadherin. Proteins of the Snail and ZEB family, E47 and KLF8, directly repress the expression of E-cadherin by binding to its promoter sequence on the DNA. Conversely, Twist, Goosecoid, Foxc2 and E2.2 indirectly inhibit E-cadherin expression. The importance of the Snail proteins is shown here, by the ability of Snail1 to activate the ZEB genes, i.e. by induction of a natural antisense transcript. Like many other signaling pathways, EMT repression is also subject to post-transcriptional regulation by microRNAs (miRNAs), increasing control possibilities on the EMT process (Thiery et al., 2009).

expression of E-cadherin in normal cells and cancer cells is regulated by several other factors that either directly or indirectly affect the expression of the main epithelial cell adhesion molecule (Figure 4).

Not all cadherin function is lost on the surface of an epithelial cell during EMT. Contrarily, the expression of N-cadherin is induced as the phenotype shifts towards a mesenchymal state, a process termed the cadherin switch, which is a hallmark of EMT (Yilmaz and Christofori, 2009). N-cadherin is normally expressed on neurons, muscle cells and vascular endothelial cells and, like E-cadherin, creates junctions by forming homodimeric bonds with N-cadherin molecules on other cells. As can be imagined, this cadherin switch has a huge impact on the adhesive properties of cells, since contact with epithelial neighboring cells is disrupted and replaced by an affinity for mesenchymal fibroblasts and vascular endothelial cells (Yilmaz and Christofori, 2009). Moreover, the shift towards N-cadherin is dominant over E-cadherin function and N-cadherin expression results in increased motility, cellular invasion and metastasis (Hulit et al., 2007).

As is clear from figure 1 and 4, the intercellular junctions involving E-cadherin are not the only contacts that have to be disrupted for a cell to detach and become metastatic. All epithelia are anchored to an underlying thin, electron dense layer of extracellular matrix (ECM), the basement

membrane (BM). Epithelial cells are connected to this layer through integrins, heterodimeric transmembrane proteins consisting of an α- and a β-subunit (Figure 3B) (Yilmaz and Christofori, 2009). Thus, for a cell to become migratory the integrin repertoire also has to reorganize and shift towards a detachment and pro-migratory set of molecules. Due to the fact that integrins are heterodimeric, cells can express at least 24 distinct intigrins, formed by a combination of 18 α-chains and 8 β-chains. Each of these combinations has a specific affinity for one or multiple ECM proteins, so the repertoire of integrins can provide cells with altered adhesive properties, due to different ligand specificities (Hynes, 1992). In the case of metastasis, integrins with a high affinity for the basal membrane are downregulated, while integrins with high affinity for the underlying ECM or tumor-associated stromal degradation components are upregulated, resulting in a cellular shift favoring motility and invasion (Desgrosellier and Cheresh, ; Hanahan and Weinberg, 2000). While epithelial cells have E-cadherin as the main adhesion molecule, integrins are more closely linked to the mesenchymal phenotype because of their interactions with the ECM and consequential migratory properties (Guarino et al., 2007). Moreover, intigrins are very dynamic adhesion molecules that can switch from low to high affinity binding states for their corresponding ligands upon binding of activators to the cytoplasmic tail of the heterodimeric molecules (Yilmaz and Christofori, 2009). The cell-ligand adhesion strength, affected by ligand concentration, integrin expression levels and binding affinity, ultimately determines if and how fast a cell migrates through the ECM (Palecek et al., 1997). Like E-cadherin, integrins are also intracellularly linked to the actin filaments of the cytoskeleton, through a complex of multiple adaptor proteins, called the IPP complex (Figure 3B) (Legate et al., 2006).

Besides providing mechanical strength via cell-matrix interactions, integrins are also important signal transducers. Signaling molecules from inside the cell can bind to the cytoplasmic tail of the αβ combinations of integrins and alter ligand affinity, while binding of the extracellular domain of integrins to ECM components results in signals transmission into the cell (Giancotti and Ruoslahti, 1999). The signaling pathways that are activated by integrin-ligand binding are crucial for cell survival, proliferation and transformation (Giancotti and Ruoslahti, 1999).

Due to downregulation of E-cadherin and adjustment of integrin expression, tumor cells can now detach from the primary tumor and, due to other EMT-mediated processes, remain viable. This gives tumor cells the opportunity to invade the underlying ECM, which is the second step in the metastatic process.

2 Invasion

All epithelial cells are supported by an underlying layer of ECM. The ECM consists of a 3D structure of proteoglycans and stromal proteins, and is constantly being remodeled. It sequesters a myriad of growth factors, chemokines and other proteins affecting cell function, which can be released and become active by the enzymatic cleavage of specific proteases (Yilmaz and Christofori, 2009). However, next to providing a binding substrate for integrins and enabling migration, the ECM and especially the dense BM also forms a barrier that has to be disrupted in order for a cancer cell to invade. In this case, after detachment, an epithelial tumor cell has to degrade the BM, adhere to the ECM by extending its surface with the proper CAMs into it, and via subsequent steps of attachment and detachment migrate towards a region of interest. In some invasive carcinomas however, the BM surrounding the tumor already is disrupted. If so, the BM will logically not form a barrier and the migrating tumor cells can invade the tissue trough the gaps in the discontinuous membrane (Rubio and Biberfeld, 1979).

To start invasion, a cell thus first has to degrade the underlying ECM structure and in most cases also a BM. The activation of matrix metalloproteases (MMPs) helps to disrupt the ECM and to clear the way for invasion (Egeblad and Werb, 2002). However, next to this physical effect of MMPs on enhancing invasion, MMPs also play a role in activating several signaling pathways. In the first place, as stated above, the substrates for integrins are altered by cleaving the ECM. Since integrin-ligand interactions result in intracellular signaling, MMPs indirectly influence these pathways (Figure 5A). In addition, MMP-mediated cleavage can expose hidden integrin binding sites (Figure 5C) or release bioactive fragments (Figure 5D). Next to providing binding substrates for CAMs, the ECM has another important function. In the matrix a myriad of growth factors (GFs) and growth factor binding proteins is sequestered, which can be released and activated by proteolytic cleavage of the ECM remodeling enzymes (Figure 5B) (Streuli, 1999).

After degradation of the surrounding matrix, tumor cells have to adhere and stretch into the underlying ECM. This requires cytoskeletal action,

which requires a proper communication between the CAMs and the cytoskeleton (Guarino et al., 2007). As stated above, both E-cadherin and the integrin family are connected to actin filaments of the cytoskeleton, and have the potential to alter cellular morphology.

The fact that E-cadherin plays an important suppressive role in EMT, greater than functioning in cellular adhesion alone, is indicated by the observations that loss of E-cadherin alone is sufficient to induce invasion and metastasis (Derksen et al., 2006; Perl et al., 1998). Moreover, restoring E-cadherin function in highly invasive epithelial tumor cell lines results in a loss of the invasive phenotype (Vleminckx et al., 1991). The key to the E-cadherin-dependent mechanisms regulating invasion lies in the ability of the cytoplasmic domain to interact with cellular modulators, of which β-catenin is the most prominent. β-catenin is crucial for establishing a connection between E-cadherin and actin filaments, an interaction necessary for cellular adhesion to occur (Kemler, 1993). Besides its role in adhesion, β-catenin is also an important intracellular signaling molecule. The functional loss of E-cadherin function liberates β-catenin from its cytoplasmic tail, and activated Wnt signaling or a non-functional destruction complex stabilizes β-catenin by preventing GSK-3-mediated phosphorylation, causing it to accumulate in the cytoplasm. β-catenin can then translocate to the nucleus and interact with the Tcf/Lef family of transcription factors and promote transcription of Wnt target genes (Clevers, 2006). These genes play important roles in development and disease and affect, amongst others, cell proliferation, invasion, migration, adhesion and morphogenesis (Yilmaz and Christofori, 2009). Not surprisingly, the E- cadherin repressors of the Snail family and Twist are also implicated to be amongst the genes affected by activation of the Wnt/β-catenin pathway. This provides more evidence for their significant role in EMT (Conacci-Sorrell et al., 2003; Peinado et al., 2007; Yook et al., 2006).

Besides β-catenin, other signaling molecules are also intracellularly coupled to the cytosolic domain of E-cadherin. Loss of E-cadherin function also releases these proteins into the cytoplasm (Figure 3A). One of these proteins is P120-catenin (P120), which, besides being part of the cytoplasmic E-cadherin complex that stabilizes junctions, interacts with regulators of Rho GTPases upon release into

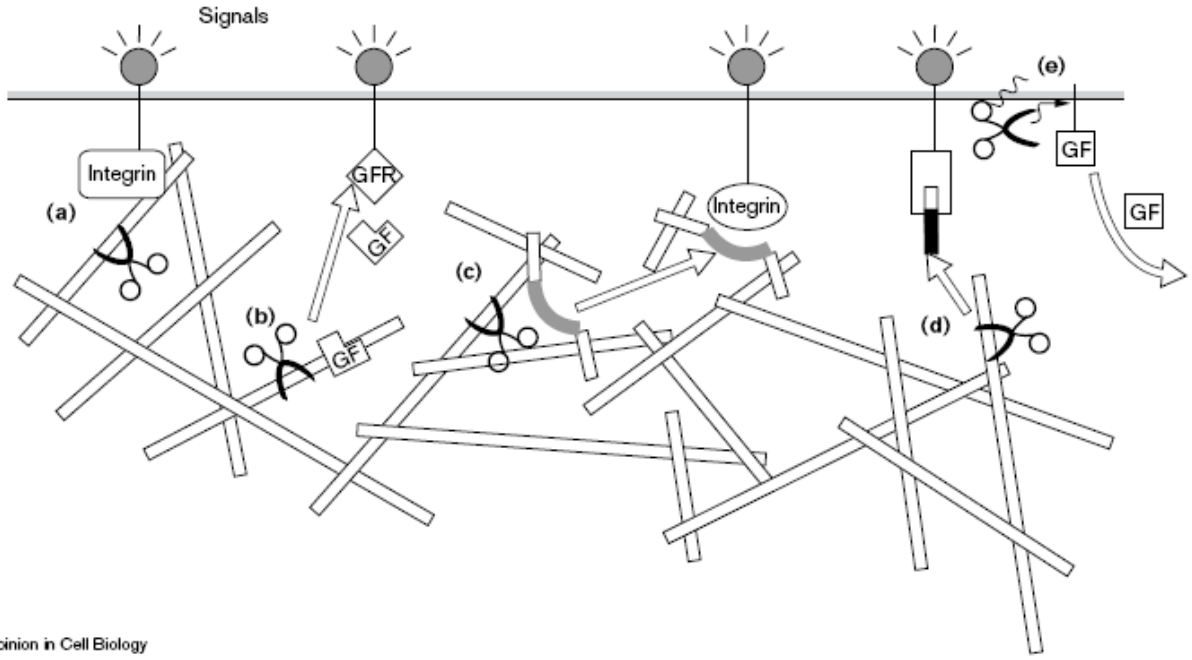

Figure 5 | MMP-mediated ECM remodeling affects EMT in multiple ways. (a) Because integrin-ECM interactions influence intracellular signaling through the cytoplasmic domains of integrin molecules, changes in the ECM can lead to the activation of different signaling pathways. **(b)** The ECM sequesters a myriad of growth factors which can be released and activated by MMPs, enabeling them to interacts with their corresponding receptor on the cell surface. **(c)** The cleavage site of the ECM can reveal new integrin binding sites (gray bands). **(d)** The cleaved ECM product can itself harbor bioactivity. **(e)** Growth factors (GFs) are sequestered in the ECM, but cells can also have inactive GFs bound to their surface, which can be activated upon MMP cleavage (Streuli, 1999).

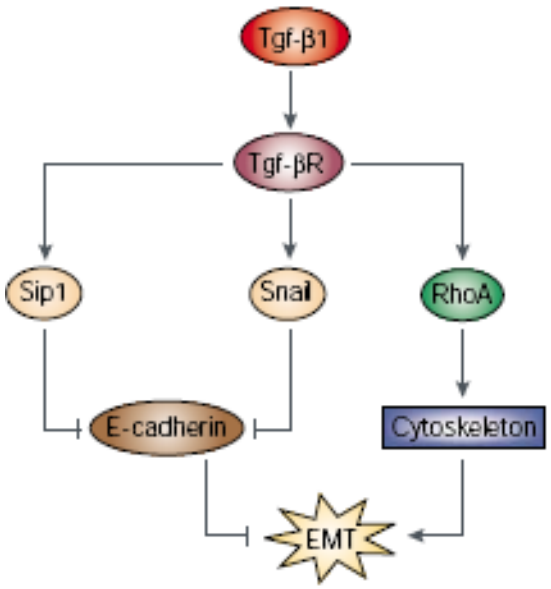

Figure 6 | The role of TGFβ during EMT. Activation of TGFβ induces EMT in multiple ways. First, by activating Snail and ZEB (Sip1) expression, E-cadherin is repressed. Second, by activating proteins of the Rho family of GTPases, the cytoskeleton is remodeled towards a migration favoring phenotype. Thus TGFβ is involved in multiple EMT-promoting pathways (Thiery, 2002).

the cytoplasm. This family of small GTP-binding proteins is involved in the organization of the actin cytoskeleton, amongst many other biological processes. By interacting with these regulators of actin filament effector proteins, free cytoplasmic P120 increases cellular invasiveness and migratory potential (Noren et al., 2000). The effects on the cytoskeleton vary between the 23 family members of the Rho GTPases, from inducing actin stress fiber formation to forming lamellipodia and filopodia. These extensions are both found on the leading edge of migrating cells and are key elements of the actin protrusive machinery (Yilmaz and Christofori, 2009).

Altogether, the E-cadherin repressors mediating the detachment step in metastasis also play important roles in enhancing the invasive phenotype of tumor cells. This effect is indirect, due to the loss of E-cadherin and the subsequent release of regulatory factors in the cytoplasm, as well as direct by influencing other EMT-related genes.

As stated above, MMP activation also releases sequestered GFs. One of the most important GFs during EMT is transforming growth factor β (TGFβ), a major inducer of developmental and pathologic EMT. Upon binding to its receptor, TGFβ induces EMT via two different signaling pathways. One requires the activation of Smad proteins, which translocate to the nucleus and regulate the expression of TGFβ target genes, including genes of the Tcf/Lef family, which are also activated by β-catenin through Wnt signaling (Nawshad et al., 2005). Furthermore, a Smad-independent TGFβ signaling pathway can activate Rho GTPases, inducing the invasive phenotype via cytoskeleton remodeling (Figure 6) (Nawshad et al., 2005).

Thus, MMP activation and subsequent release of GFs (Figure 5E) has a significant impact on multiple biological processes in the cell. Moreover, MMPs have other cleaving products, among which multiple CAMs. E-cadherin is probably the most important during EMT. The cleavage fragment is extracellularly released, and since E-cadherin molecules provide adhesion by binding to other E-cadherin molecules, the fragments can bind to and interfere with endogenous E-cadherin function (Noe et al., 2001). Furthermore, besides affecting integrin ECM ligands, the integrins *per se* are also cleaved by MMPs, i.e. MMPs are involved in the processing of inactive precursor subunits to mature and actively functioning integrins (Deryugina et al., 2002).

The expression of MMPs by cells undergoing EMT is regulated by similar transcription factors as discussed above. The Snail proteins have been shown to upregulate the expression of multiple members of this family of proteolytic enzymes (Jorda et al., 2005; Miyoshi et al., 2004), just like ZEB transcription factors (Thiery et al., 2009). Next to Snail-mediated activation of MMPs, MMPs can also induce Snail expression, a process in which the MMP alone is sufficient to start EMT, underscoring the importance of these proteases in the tumor microenvironment (Radisky et al., 2005). The E-cadherin repressors also indirectly increase MMP production, as released cytoplasmic β-catenin has been shown to strongly increase the expression of an MMP family member in intestinal carcinomas (Crawford et al., 1999).

Now that the cells are detached from each other and the ECM, and the ECM is properly remodeled for invasion, new adhesive contacts have to be made to enable the cell to pull itself forward. As discussed above, fully mesenchymal cells express no E-cadherin, and integrins are the main CAMs on migratory mesenchymal cells to provide flexible cell-matrix interactions necessary for invasion. Invasion starts with polarization of the cell, forming the previously mentioned actin-rich lamellipodia and filopodia, that form protrusions of the plasma membrane at the invasive edge. The lamellipodia, cellular tools for locomotion, can interact with the

ECM through the accumulation of CAMs, i.e. integrins (Wang et al., 2008). However, next to the adhesive functions of integrins, their signaling and recruiting functions are also crucial during EMT. β1-integrins, for example, are known to mediate the recruitment of an MMP family member, which in its turn is able to activate latent ECM-bound TGFβ (Yilmaz and Christofori, 2009). Most importantly for EMT, however, are the signaling pathways that are affected by the different integrins. Cells that detach from the epithelial layer under physiologic conditions undergo apoptosis to prevent them from homing at inappropriate locations. This process is referred to as 'anoikis', and tumor cells that go through EMT must obviously prevent this type of cell death (Giancotti and Ruoslahti, 1999). Intracellular signaling upon integrin-ligand interactions can provide these survival signals and cancer cells often switch their integrin expression pattern towards an oncogenic stimulating repertoire (Yilmaz and Christofori, 2009). Next to the integrins, N-cadherin also localizes to the lamellipodia, which, as discussed above, is also involved in increased motility and invasion (Comunale et al., 2007).

After a cancer cell has detached from the primary tumor and obtained its motile properties, it migrates towards a blood- or lymph vessel, enabling itself to travel through the circulation and colonize a distant site. Filopodia function as sensory organs during this process, leading the way towards vessels through the ECM (Yilmaz and Christofori, 2009). Formation of the filopodia is regulated by Rho GTPases and the Wnt signaling pathway, which targets proteins involved in actin bundling during the development of filopodia, i.e. Fascin (Yilmaz and Christofori, 2009). Not surprisingly, during cancer progression increased Fascin expression is associated with a poor prognosis (Hashimoto et al., 2004).

The signals that attract the cancer cells and stimulate them to migrate in response to a gradient of soluble factors vary, but epidermal growth factor (EGF)-like factors, secreted by macrophages or formed during ECM proteolysis, are key chemoattractants, indicated by the increased

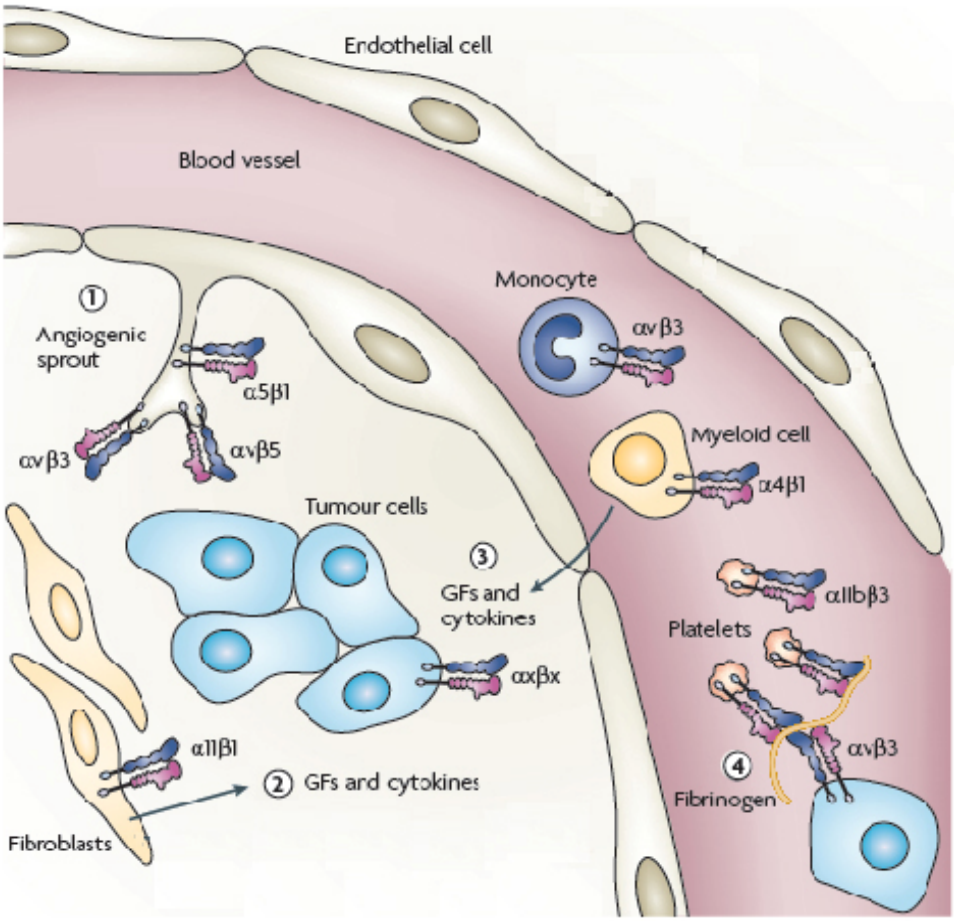

Figure 7 | Integrin interaction during entry into the circulation. Integrins function in multiple processes to assist immune cells and tumor cells in entering, surviving and leaving the bloodstream. (1) Integrin expression on endothelial cells is required for angiogenesis, providing growing tumors with oxygen and nutrients, while exporting waste products away. (2 &3) Fibroblasts and other host (immune) cells present in the tumoral microenvironment and bloodstream secrete growth factors and cytokines, attracting leukocytes and potentially tumor cells from the circulation and aid intravasation and extravasation, i.e. by increasing vascular permeability. (4) Once in the circulation, tumor cells can interact with platelets through their integrins, which prevents immune detection and aid in tumor cell arrest, after which the cells can extravasate and colonize a new region (Desgrosellier and Cheresh, 2010).

expression of the EGF receptor (EGFR) on metastatic tumor cells (Condeelis and Segall, 2003). Other chemotactic factors guiding migration of cancer cells towards the vasculature include fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), sphingosine-1-phosphate (S1P), and many others (see Table 1 of Wang et al., 2002), predominantly expressed by immune cells, platelets and smooth muscle cells (Condeelis and Segall, 2003; Kume and Gimbrone, 1994; Peoples et al., 1995; Wang et al., 2008). The end result of all these factors is analogous: creating a gradient to which the cancer cells respond by extending lamellipodia-like structures, followed by movement, towards the location containing the highest concentration of the chemoattractant, i.e. the vasculature (Bailly et al., 1998). As soon as the migrating tumor cells have reached the vasculature, they face the next barrier: entering the bloodstream.

3 Intravasation and extravasation

Intravasation is a critical step for metastasis and requires cooperation of both tumor cells and endothelial cells lining the vessel. Much about transendothelial passages has been learned by studying leukocytes responding to inflammatory signals. Cancer cells may even mimic the mechanisms that leukocytes use, and again proteolytic and cellular interactions play key critical roles in these processes (Buxton et al., 2010; Chambers et al., 2001). Moreover, next to possibly mimicking leukocytes, some leukocytes are recruited by the primary tumor to the tumor microenvironment, for example macrophages, which subsequently directly help tumor cells to cross the vascular endothelium (Figure 7). A paracrine loop, involving chemokines secreted by both macrophages and cancer cells, EGF and CSF respectively, is an important mediator of intravasation (Condeelis and Segall, 2003). Next to secreting factors that guide tumor cells to the bloodstream, there also seems to be a spatial relationship between the sites where immune cells exit the vasculature and cancer cells enter the circulation. Specific sites could thus be used for transendothelial migration of the immune system and tumor intravasation (Madsen and Sahai).

However, here I will mainly focus on the events that occur within the cancer cells themselves, enabling them to intravasate. As stated before, again cellular interactions play an important role in intravasation, so the CAM expression pattern of cells arriving at the endothelial lining is pivotal for transendothelial migration. However, even before the invasive tumor cells reach the endothelial lining, a final basement membrane has to be crossed, separating the vessels from the stroma. Similarly to the epithelial BM, MMPs are important for degradation of this final barrier. Especially MMP-9 has shown to be required for intravasation (Kim et al., 1998), which is under control of Snail expression (Jorda et al., 2005).

N-cadherin is one of the CAMs that facilitates migration over the endothelium. Since the endothelium expresses N-cadherin too, homophilic interactions between the endothelial cells and N-cadherin bearing tumor cells are made possible and subsequently these cells can migrate into the vessel (Hazan et al., 2000). This observation is in agreement with the data indicating that high levels of Twist stimulate cancer cells to enter the vasculature (Yang et al., 2004). Twist, next to being a potent inhibitor of E-cadherin, namely also increases the expression of N-cadherin (Yang et al., 2007). Hence, the Twist-mediated cadherin switch favors intravasation by enabling cells to interact with the outer lining of the vessels trough N-cadherin. However, others report that although N-cadherin is required for the transendothelial passage, it is not sufficient (Drake et al., 2009).

Interestingly, E-cadherin might also play a role in intravasation. Cowley and Smith reported that in 40% of the adenocarcinomas they examined, the E-cadherin expression of the tumor cells was raised in the tiny intravascular space, compared to the adjacent much larger extravascular part of the carcinoma (Cowley and Smith, 1995). Hence, entrance of tumor cells into the vasculature is in some cases associated with upregulation of E-cadherin, while exit into the stroma at a secondary site associates with E-cadherin downregulation (Smith and Pignatelli, 1997).

Next to the cadherin family, also the other main family of CAMs discusses above, the integrins and associated molecules, is involved in intravasation, although the amount of reports demonstrating the requirement of integrin-mediated interaction to the vascular endothelium to enhance entrance into the blood stream is marginal. One recent report does show, however, that blocking the integrin-associated tetraspanin CD151, a transmembrane protein interacting with the integrin α-subunit and thereby regulating integrin function, prevents access to the vasculature (Zijlstra et al., 2008). A more profound role of integrin function in the process of intravasation is probably the ability of some members of this family of CAMs to promote angiogenesis (Figure 7). In order for a solid tumor to grow, the cancer cells rely on the formation of

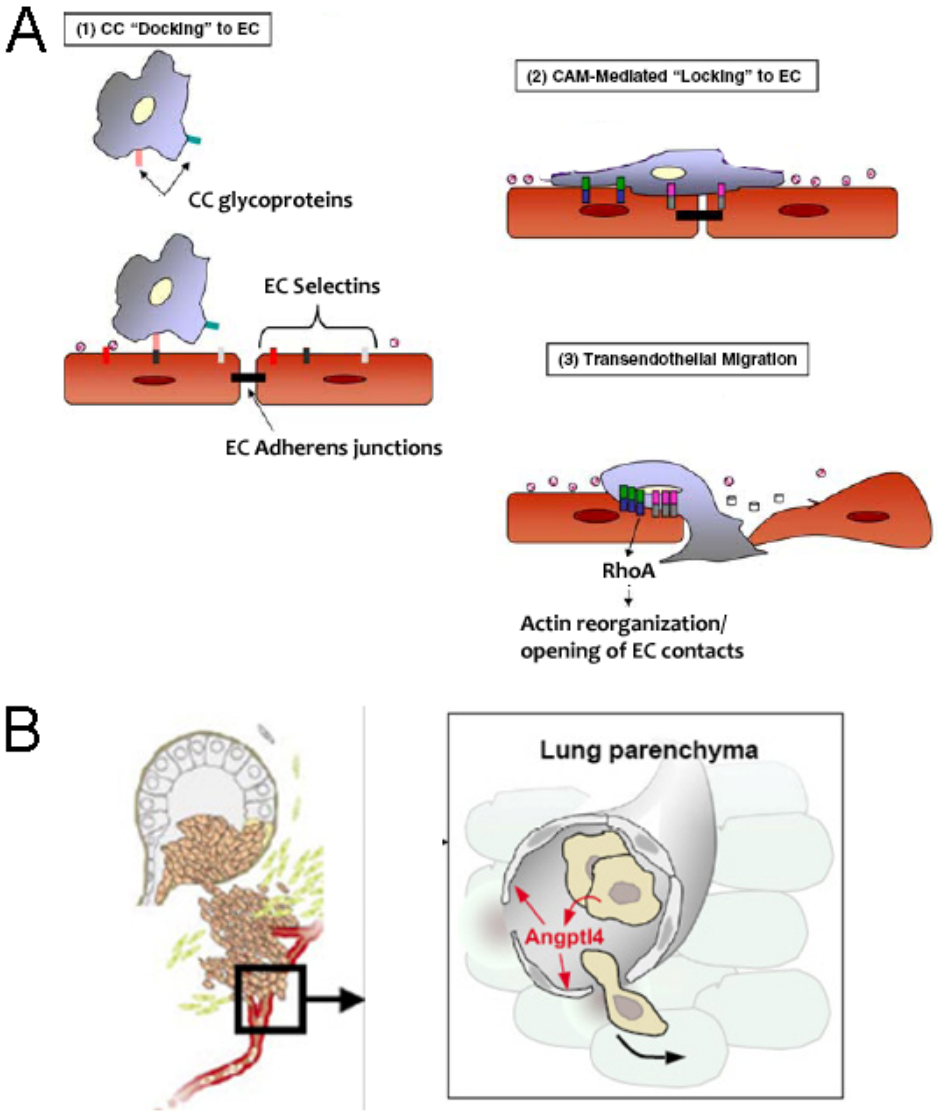

Figure 8 | Mechanisms of cancer cell extravasation. (A) Cancer cell (CC) extravasation based on selective docking to endothelial cells (ECs), mediated by several CAMs. Similar to leukocytes, the cancer cells dock to the endothelium (1), roll along the vessel wall (2), arrest and extravasate (3). A myriad of CAMs facilitates the different steps of the CAM-based extravasation theory, where adhesion is stimulated by cytokine expression in the microenvironment (see text for details). The clustering of the CAMs can also lead to activation of the Rho family of GTPases, leading to cytoskeletal reorganization and subsequent exiting of the vasculature (Adapted from Miles et al., 2008). **(B)** The second mechanism of extravasation starts with aggregation of the cells in the capillaries. Since cancer cells are much larger than leukocytes, they are much more prone to become trapped in small vessels (left panel). After becoming trapped however, the cells still need to extravasate. One method of leaving the vascular is the expression of factors that disrupt the endothelial wall, i.e. angiopoietin-like 4 (Angptl4), stimulated by TGFβ activity (Adapted from Padua et al., 2008).

new blood vessels to meet their metabolic needs. This process, angiogenesis, thus supports tumor growth by providing the proliferating cells with oxygen and nutrients, while cellular waste products are disposed (Tlsty and Coussens, 2006). Sustained angiogenesis is thus required for tumor growth and considered to be one of the hallmarks of cancer (Hanahan and Weinberg, 2000). Factors secreted by (immune)cells from the tumor microenvironment or released from the stroma can promote angiogenesis (Tlsty and Coussens, 2006). Recent studies have shown that upregulation of certain integrins on the endothelial cells is key to regulating the sprouting of new vessels in the peritumoral space (Avraamides et al., 2008). Like on other cell types, integrin signaling in the endothelial cells of the vascular wall leads to cell migration, by providing cells with a CAM able to bind ECM components in the tumor microenvironment, and enhanced survival characteristics during tumor cell invasion, leading to the formation of new vessels, increasing the chance of metastasis (Avraamides et al., 2008). In addition, the newly formed vessels are abnormal with respect to their organization, structure and biology, compared to normal blood vessels. Harold Dvorak, who was the first to propose that tumors induce angiogenesis by activating wound healing programs, stated that 'tumors make bad blood vessels' (Ribatti, 2007). The nature of these 'bad' vessels, enables cancer cells to enter the circulation easily, i.e. the vessels are twisted and the endothelial cells do not overlap nicely, rendering them leaky, and hence facilitating

intravasation {Desgrosellier, 2010 #38}. This vascular permeability could be due to the expression of specific integrin family members on the endothelial cells, thereby promoting metastasis {Avraamides, 2008 #81}.

The main factors stimulating this integrin-mediated growth of new vessels are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and TNFα. Next to the recruited macrophages, these factors are also secreted by the tumor cells themselves. VEGF expression has been detected to be under the control of Snail. Moreover, both E-cadherin repressors Snail and E47 have been shown to induce several pro-angiogenic factors (Peinado et al., 2004a). VEGF and FGF signaling on their turn can promote EMT by stimulating the expression of the Snail family and Twist (Wanami et al., 2008; Yang et al., 2006). Thus, a regulatory loop between angiogenesis and EMT is suggested to contribute to tumor progression (Thiery et al., 2009).

Now that the tumor cells have entered the blood stream and before they exit the circulation, they are exposed to a totally different microenvironment, facing high doses of shearing stress due to blood flow. Non-invasive cells, or cells crossing the endothelium slowly, are observed to fragment during intravasation (Wyckoff et al., 2000). Thus tumor cells must protect themselves against shearing in order to survive in the circulation. One way by which tumor cells can achieve this is by remodeling the cytoskeleton and indeed, the authors observed differential expression of Rho family members and consequent changes in cytoskeleton conformation in metastatic cells capable of survival in the bloodstream (Wang et al., 2002). Other means by which cells are protected is the expression of integrins. Next to interacting with the ECM, some integrin members can interact with platelets and leukocytes circulating in the blood. These interactions mediate cell arrest and protect the tumor cells from the shearing forces (Felding-Habermann et al., 2001). Additionally, platelets can hide the tumor-associated antigens on the cancer cells, thereby preventing immune cell recognition in the blood and subsequent elimination (Figure 7) (Palumbo et al., 2005). Next to platelets, intravenously injected cancer cells expressing MMP14 also have an increased survival rate, compared to similar cells not expressing this protease (Tsunezuka et al., 1996). The cells that have survived their new hostile microenvironment now face the next barrier before colonizing a new location; extravasation. Although the process of extravasation is studied more extensively than intravasation, the exact mechanisms are unclear. The main debate is whether or not cancer cells selectively adhere to the endothelium with their CAMs (Kobayashi et al., 2007) or that the cells simply become trapped, which, combined with an invasive potential, results in migration out of the blood vessel (Sikes et al., 2004). The CAM-theory of cancer cell extravasation is conceptually similar to leukocyte diapedesis through the vasculature, a widely studied process. The leukocytes first loosely bind to the endothelium (1), roll along the vessel wall (2), arrest by firmly adhering to the vascular wall (3) and are then ready to cross the vessel and underlying BM (4) (Figure 8A) (Ley et al., 2007). It is no surprise that leukocyte exiting the vasculature demand the expression of specific CAMs, and cancer cells have been shown to express similar adhesion molecules (Miles et al., 2008). Although the first steps of leukocyte transendothelial migration, docking to the endothelium and rolling, is controversial in cancer cell extravasation, cancer cells have been shown to express selectins, the cell adhesion molecules mediating the rolling, and even rolling has been observed, albeit less frequent (Figure 8A) (Giavazzi et al., 1993).

The next step however, locking to the endothelium, is a substantially proven process and integrins play an important role herein. $\beta 1$ integrins can interact with members of the VCAM (vascular cell adhesion molecule) family, expressed by endothelial cells (Klemke et al., 2007). Other integrins have also been implicated in endothelial interaction preceding transendothelial migration, i.e. the $\beta 3$ subunit (Voura et al., 2001). Integrin-mediated binding to the endothelium is not the only interaction stimulating extravasation and in some cases crossing the endothelium is even not necessary to exit the blood vessel. In these cases, the integrins directly bind to exposed ECM underneath the vessel (Wang et al., 2005). This occurs in organs containing a discontinuous endothelium, i.e. the liver. Here, integrins can directly contact the ECM from the blood vessel, and assist extravasation without interacting with the endothelium (Rosenow et al., 2008).

As described above, the importance of intracellular signaling through integrins also plays an important role in extravasation. Similar to invasion, the cancer cells have to squeeze themselves through the endothelial barrier, requiring remodeling of the cytoskeleton. Integrin-mediated activation of Rho GTPases is thus also a critical process for entering a secondary site which is extensively shown in leukocyte extravasation (Ridley, 2001). Fewer reports prove integrin signaling pathways activating Rho-mediated actin remodeling in cancer cells, but it becomes clearer

that this indeed seems to be a plausible hypothesis, since Rho GTPases are critical for transendothelial migration (Miles et al., 2008).

As already mentioned earlier in this chapter, endothelial cells express N-cadherin. Besides intravasation, homophilic N-cadherin interactions can also be used by tumor cells to extravasate, where repressing N-cadherin expression leads to an inhibition of transendothelial migration (Qi et al., 2005), however others have reported that N-cadherin expression alone is not sufficient for extravasation (Drake et al., 2009). Moreover, Drake *et al.* show that the EMT-inducing transcription factor ZEB1 is required for transendothelial migration in PC-3 tumor cells, highly metastatic cells derived from a prostate adenocarcinoma. They claim that ZEB1 allows for N-cadherin mediated extravasation by activating other cellular factors repressing the epithelial phenotype (Drake et al., 2009).

Just like during intravasation, vascular permeability also aides in tumor cell extravasation. Cells extravasating at sites where previously factors like VEGF, TNFα (see above) and other inflammatory signals have been secreted, extravasation is similarly facilitated by this increased permeability (Madsen and Sahai). Even more, next to factors from the extravascular microenvironment, factors secreted by the cancer cells themselves also disrupt endothelial cell-cell contacts. TGFβ expression, previously described as a potent inducer of EMT, also activates other factors, mediating vascular permeability and thereby enabling extravasation into the lungs (Padua et al., 2008). Besides TGFβ, VEGF also disrupts endothelial cell-cell interactions (Lee et al., 2003). Thus, potentially Snail-mediated expression of VEGF also enhances transendothelial migration by downregulating endothelial integrity.

The previously described methods that cancer cell can use to extravasate are all based on the selective binding of CAMs to the vasculature, followed by a vascular exit at a location of preference, a mechanism similar to that of leukocytes. However, leukocytes are much smaller than cancer cells, and in many cases the arrest of disseminated cells in a blood vessel is simply due to the size restrictions of the cells (Morris et al., 1993). After becoming trapped however, the cells still have to interact with the endothelium to enable the transendothelial migration (Figure 8B).

As a last step, after overcoming the endothelial barrier, similarly to entry into the blood, a basement membrane has to be crossed. Because the mechanisms used to solubilize this BM do not differ significantly from the mechanisms during invasion and intravasation, I will not discuss this further here and refer to the previous chapters.

4 Colonization

All the previous steps combined have resulted in the escape of a tumor cell from the primary tumor to the arrival of the cell at a secondary site in the organism. The final step to complete the metastatic cascade is colonization, the proliferation of the tumor cell and the formation of a secondary tumor. Like in all the previous steps, the proper growing of a secondary tumor depends on multiple cellular properties next to proliferation, i.e. preventing apoptosis and initiating angiogenesis. Hence, the interaction with the local microenvironment is of great importance to the success of a tumor cell to colonize. This is not a new concept; already in 1889 the English surgeon Stephen Paget described the phenomenon of the non-random formation of metastasis, and postulated his ‘seed and soil’ hypothesis (Paget, 1889). The seed, a metastatic cancer cell, can only grow when it arrives at a soil, local microenvironment, supporting the growth requirements of the seed. This proper soil is not always reached by the traveling cancer cells. Even more, colonization is known to be a very inefficient event and in an experimental melanoma model only 1 in 100 injected tumor cells was capable of forming micrometastases after 10 days (Steeg, 2006). Thus, the local microenvironment the metastasized cancer cell arrives in greatly determines metastatic outcome. This has been shown on a matrigel culture with PC-3 cells. When these cells are cultured in planar dishes, they resemble cells that have undergone EMT; no E-cadherin is expressed and mesenchymal markers are expressed by the cells, i.e. Vimentin. Thus, no epithelial junctions are formed and the cells live solitary and non-polarized. On a 3D matrigel culture however, cell-cell contact between the cells was restored, prostate-specific markers increased while Vimentin expression decreased. Together with the observation that the cells formed hollow acinar spheroids, this was an indication that the epithelial phenotype was restored and thus indicates the influence of the microenvironment on cell state, and in this case the induction of MET (Lang et al., 2001).

Multiple factors in the new microenvironment affect the growth of the arrived cancer cells, and influence multiple important pathways including CAM expression, cytokine expression and growth factors (Tlsty and Coussens, 2006). In these interactions with the tumor cells, termed the stromal

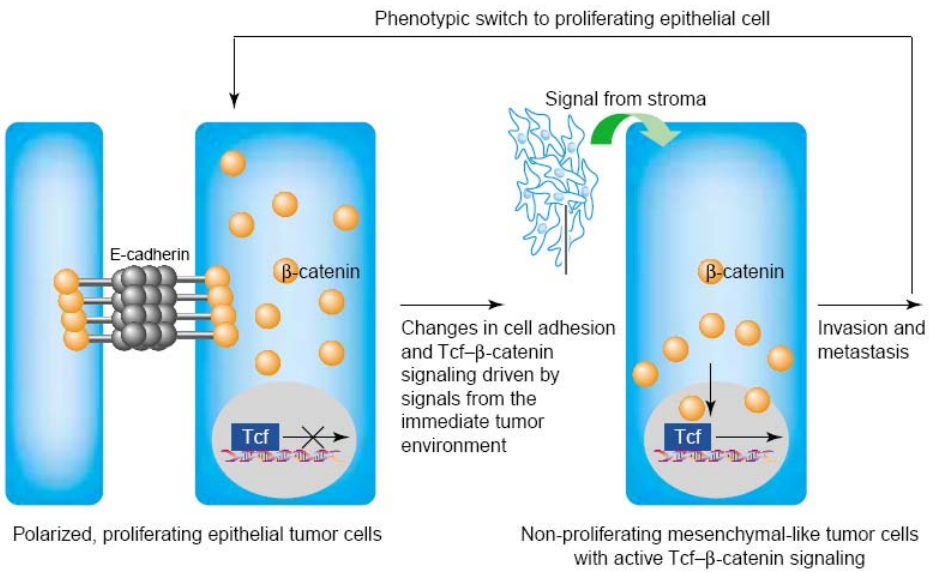

Figure 9 | The EMT to MET switch during tumor cell colonization and E-cadherin expression. As the tumor cells that had undergone EMT, giving them migratory potential, arrive at their new location, a switch back to their original epithelial phenotype is observed. This process, termed MET, is likely initiated by the new microenvironment and encompasses the re-expression of E-cadherin and the subsequent translocation of β-catenin to the membrane surface. This results in blocking of Tcf/Lef signaling and tumor cells regaining proliferative potential, enabling the forming of a secondary tumor mass (Adapted from Barker and Clevers, 2001).

reaction, the inflammatory response and recruited cells from the immune system play crucial roles. Next to the earlier mentioned role of macrophages in the transendothelial passage of cancer cells and secreting growth stimulating factors, the main proinflammatory transcription factor nuclear factor κB (NFκB) is also of great importance. This TF has been shown to enhance tumor growth in at least two ways. Firstly, NFκB prevents cell death of cells with malignant potential. Secondly, its expression stimulates the expression of proinflammatory cytokines in immune cells in the tumor microenvironment, promoting proliferation and survival (Tlsty and Coussens, 2006). Moreover, NFκB has been observed to regulate Snail1, Snail 2 and Twist, thereby affecting EMT and/or MET (unpublished observations) (Klymkowsky and Savagner, 2009; Tlsty and Coussens, 2006).

Next to the altered microenvironment the disseminated cancer cells arrive in, the cells themselves undergo a transformation too. As stated in the introduction, the tumor cells found at the metastatic locus resemble the cells in the tumor they arose from. Hence, the cells that have undergone EMT to start the metastatic cascade, now switch to MET programs to regain their original phenotype. This shift back towards the epithelial phenotype reduces the migratory properties of the cell, but induces the proliferative potential, important for colonization (Brabletz et al., 2001; Kalluri and Weinberg, 2009). This implicates a reverse in the expression of the transcription factors previously discussed to induce EMT. Indeed, already during formation of the somites during embryonic development, the repression of, for example, the Snail genes leads to somite epithelialization, an MET (Dale et al., 2006). Similar to the signals previously described to induce EMT, i.e. Wnt and TGFβ, signals from the new microenvironment are likely to promote the transition back to epithelia (Kalluri and Weinberg, 2009).

In regaining their original epithelial character, suppressed molecules to enhance epithelialization are expressed again. E-cadherin, for example, is found to be re-expressed during MET in kidney formation (Kuure et al., 2000). Also during cancer progression, cells that undergo MET are shown to have retrieved normal membranous E-cadherin levels. This allows the cells to aggregate, providing better survival chances for the proliferating cells (Brabletz et al., 2005). Furthermore, next to connections with other tumor cells, also connections with the host cells are made through E-cadherin. This results in the activation of intracellular signaling pathways, providing 'home' signals to the cells, thereby stimulating proliferation (Wells et al., 2008). The transcriptional regulation of this E-cadherin switch is probably associated with differential expression of Snail, which due to its highly unstable nature is extremely dynamic (Guarino et al., 2007). Next to being a repressor of E-cadherin, the enhanced invasiveness induced by Snail inhibits proliferation and protects against apoptosis during migration (Barrallo-Gimeno and Nieto, 2005). As the tumor cells reach their new location, Snail expression is downregulated, which

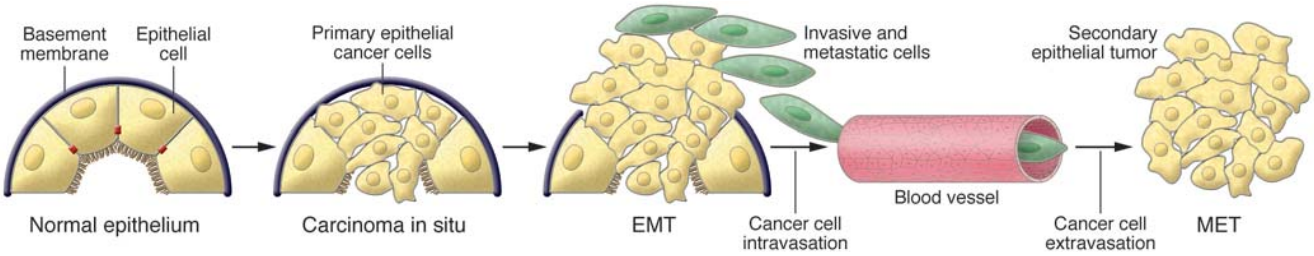

Figure 10 | EMT during the different steps of the metastatic cascade. As normal epithelial cells start to become malignant, EMT programs are activated and the cells lose important CAMs and detach from the epithelial sheet. The newly acquired mesenchymal characteristics also include increased migratory potential, so the metastatic cells can invade the surrounding tissue. As a blood vessel is reached, intravasation takes place and the cells are transported and extravasate at a secondary site in the organism. Signals from the new microenvironment subsequently activate a reverse transition, MET, to regain the epithelial and proliferative phenotype in order to grow a secondary tumor mass (Adapted from Kalluri and Weinberg, 2009).

favors the epithelial and proliferative phenotype (Brabletz et al., 2005).

Next to Snail, also β-catenin has a role in MET, maybe not surprisingly since its earlier mentioned intracellular association with the cytoplasmic domain of E-cadherin. When E-cadherin is lost, the membrane-localized β-catenin translocates to the nucleus, activating Tcf/Lef genes, thereby reducing proliferative potential. Thus, by re-expressing E-cadherin, β-catenin becomes membrane-associated again, which results in a tumor cell with an increased proliferative activity (Figure 9) (Brabletz et al., 2005). Besides β-catenin, other players from the Wnt pathway also regulate Snail expression during colonization. Activation of the kinase GSK-3, for example, can degrade the unstable Snail protein (Zhou et al., 2004). Since initiation of growth appears to be the main rate-limiting step during the metastatic cascade, this regulation of Snail expression and the cellular plasticity to switch between EMT and MET it brings forward, is crucial for a cell to complete metastasis (Hugo et al., 2007).

So what other colonization favoring effects do these changes in transcription factor expression levels exert? For one, the MMPs that functioned in the last phase of the extravasation step, are also important to promote colonization (Chambers et al., 2001). The GFs that are released by proteolytic cleavage can stimulate proliferation of the tumor cells at the secondary site (for review see Egeblad et al., 2002). Furthermore, preventing apoptosis is another key characteristic the disseminated tumor cells must own. Although the different members of the MMP family have different effects on apoptosis, some of them are known to have a negative effect on tumor outcome by inhibiting apoptosis (Egeblad and Werb, 2002).

Finally, after having overcome all previously described obstacles by activation of multiple metastasis-inducing transcription factors, the tumor cells have now completed their metastatic journey and formed a secondary tumor at a distant site.

Discussion

It is clear from literature that EMT has important functions during development and disease. Type 1 and type 2 EMT both serve crucial roles during development and wound healing, respectively. Type 3 EMT, however, has no physiological functions and is used by cancer cells to escape the primary tumor, migrate to the local vasculature to be transported throughout the body, and exit the vessel at a distant site. MET programs then revert the cell back to its original epithelial phenotype, and due to the initiated proliferation, a secondary tumor can arise (Figure 10). During these transitions between epithelial and mesenchymal states, several transcription factors and signaling pathways play important roles and some of the key molecules they affect are those involving cell adhesion and cytoskeletal reorganization. The best studied and probably most important transcription factors during EMT are Snail1 and Snail 2, ZEB1 and ZEB2, Twist and E47, all repressors of the main epithelial cell adhesion molecule E-cadherin (Figure 11). In fact, E-cadherin is seen as one of the most indicative molecules during EMT, since its loss of expression in cancer cells is the first of an early step of EMT, after which the cells can detach and are able to migrate and invade. Although all the previously mentioned transcription factors negatively regulate E-cadherin, their other functions and how they are interrelated is not yet clear. Peinado *et al.* have proposed a model for the different TFs during EMT. According to this model, Snail1 and ZEB2 play a role in the first step of tumor progression, thereby initiating the invasive process. Snail2, E47 and

ZEB1 subsequently are involved in the maintenance of the malignant phenotype. Twist, finally, has a role in stimulating intravasation (Peinado et al., 2004b). However, the manifestations of TF activation again differ between cell types and similar TFs can have different effects in different cell types (Drake et al., 2009). Examining the exact role of each transcription factor is complex because of the diversity of factors that influence EMT from outside the cell, and the different regulatory functions a single EMT-transcription factor can have within a cell (Figure 12).

The popularity of the theory that EMT is required for metastasis can be seen when 'epithelial-mesenchymal transition' and 'cancer' are searched for on PubMed (NCBI, NIH), which yield more than 930 references (accessed 09/17/2010). However, not all literature agrees on the biological existence of the transition during carcinogenesis, the main arguments being that there is a lack of convincing evidence of EMT *in vivo* and the absence of EMT-specific markers to detect cells that have undergone EMT, to distingsuish them from regular mesenchymal cells, i.e. fibroblasts (Tarin et al., 2005). The first problem with EMT lies in the fact that distant metastases, rather than still being mesenchymal, are histopathologically similar to the epithelial tumor they derived from. To explain this in line of the EMT hypothesis, a reverse transition, MET, was postulated (Thiery, 2002). However, experimental data in compliance with the MET hypothesis during carcinogenic progression is limited (Chaffer et al., 2006). On the other hand, the use of new experimental procedures has shown evidence for the existence of EMT in breast cancer *in vivo*, using mouse mammary tumor models (Trimboli et al., 2008). Although this research only indicates involvement of EMT in breast cancer metastasis, it does suggest a role for EMT in tumor progression in general, and increases the likeliness that at a distant location, the cells undergo MET. To really prove the EMT-MET hypothesis however, it is necessary to mark individual cells in the primary tumor and track them during the metastatic process, enabling live detection of any changes in cell state. Microscopic techniques that allow us to do this, are described later in this discussion.

The fact that disseminated tumor cells in, for example, the lungs express epithelial markers, of which the most important one is E-cadherin, suggests that these cells re-express the CAM, but it can also indicate that E-cadherin has been expressed throughout the metastatic process and that tumor cells detached from the primary tumor as E-cadherin positive cells. Even though most

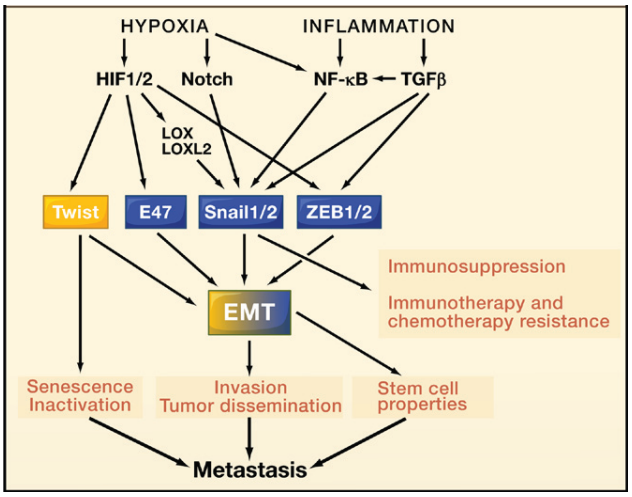

Figure 11 | Environmental factors and their effect on EMT-inducing transcription factors. In the middle the six most important transcription factors during EMT are shown. Next to being repressors of the CAM E-cadherin, all six also have other metastasis-enhancing properties, either directly or via EMT pathways. In the microenvironment, hypoxia and inflammatory responses have crucial effects on cancer cells and can stimulate EMT through one or more of the shown transcription factors (Adapted from Thiery et al., 2009).

migratory cells are found not to express E-cadherin, due to the great heterogeneity in most tumors some E-cadherin expressing cells could have escaped the primary mass (Wells et al., 2008). The expression of E-cadherin all along the metastatic process has also found to be positively affecting other migratory properties. In the bloodstream for example, E-cadherin mediated clustering of cells could enhance the formation of a tumor cell clump, which subsequently gets trapped easier and can start growing at a secondary site, since the tumor cells already have the proliferative phenotype (Guarino et al., 2007). Moreover, some experiments show that the levels of E-cadherin fluctuate more between the different steps of metastasis than just off at detachment, and on again at colonization. Dependent on the microenvironment, it is reported i.e., that upregulation of E-cadherin facilitates entrance into an intravascular compartment, whereas E-cadherin downregulation is linked to its subsequent exit out of the vasculature (Smith and Pignatelli, 1997). Thus, the expression of E-cadherin, the key CAM during EMT, might not be so black and white in different tumors during carcinogenesis as is described by the standard EMT hypothesis.

Next to the possibility that either E-cadherin positive or negative cells metastasize or that single cells differentially express E-cadherin throughout the different metastatic steps, a new role of EMT

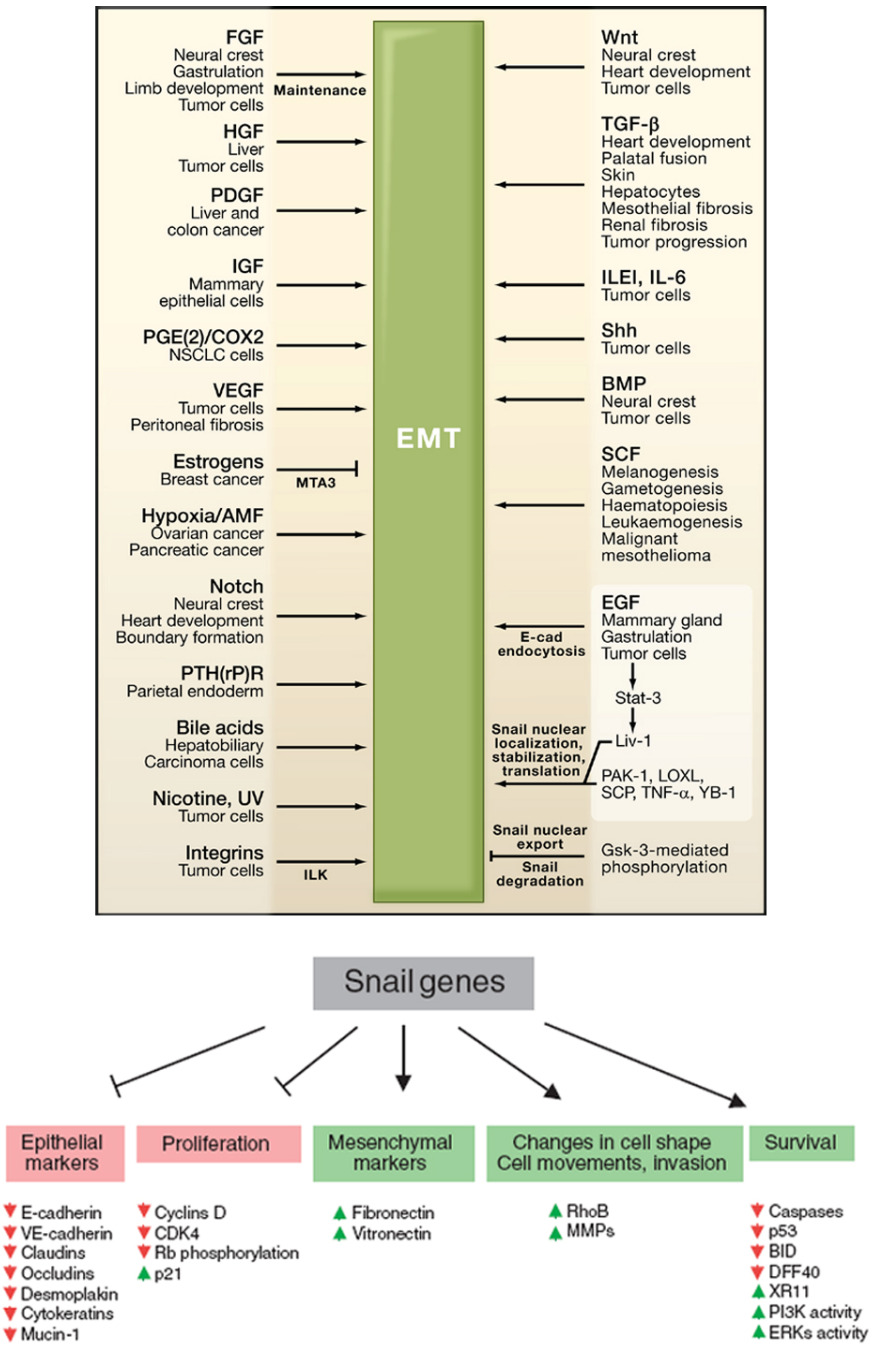

Figure 12 | EMT-inducing signaling pathways and downstream targets of Snail genes. The upper panel shows the wide variety of extracellular agents known to induce EMT, both developmental as well as during carcinogenesis (Thiery et al., 2009). The lower panel shows the many different effects a single gene involved in inducing EMT, in this case Snail, can have on a cell. The downstream targets range from decreased epithelial markers and proliferation, to increased mesenchymal markers and survival (Barrallo-Gimeno and Nieto, 2005).

has recently been proposed (Tsuji et al., 2009). According to this theory, cooperation between epithelial and mesenchymal cancer cells induces metastasis, thereby also ruling out a role for MET. The authors found EMT to be induced through Twist2 in a keratinocyte cell line. Neither control cells nor the EMT-induced cells were able to form lung metastasis after being subcutaneously injected in a xenograft model. Both did form primary tumors, of which only the EMT-induced cells invaded the local tissues and intravasated in the local vasculature (Tsuji et al., 2008). When both cell types were intravenously injected, only the control cells that had not undergone EMT were able to establish metastasis in the lung. Interestingly, when a 1:1 mixture of EMT cells and non-EMT cells was injected subcutaneously, both cells types were able to invade and intravasate and the cells that had not undergone EMT formed lung metastasis (Figure 13) (Tsuji et al., 2008). The authors conclude that EMT is required for metastasis, to give tumor cells the capabilities to degrade the extracellular matrix and 'lead the way' for the non-EMT cells into the vasculature. The non-EMT cells subsequently are the cells that extravasate and form metastasis (Tsuji et al., 2009). According to this theory, EMT cells are thus not able to extravasate, probably because of their loss in adhesion molecules, i.e. E-cadherin that was earlier described to have a function in exiting the vasculature (Smith and Pignatelli, 1997). On the other hand, the extravasation of the cells is in the lungs, where due to the many small capillaries trapping of the cells is a well possible first step of extravasation. Since the cellular morphology changes in the cells undergoing EMT, it could be possible that the non-EMT cells get stuck more

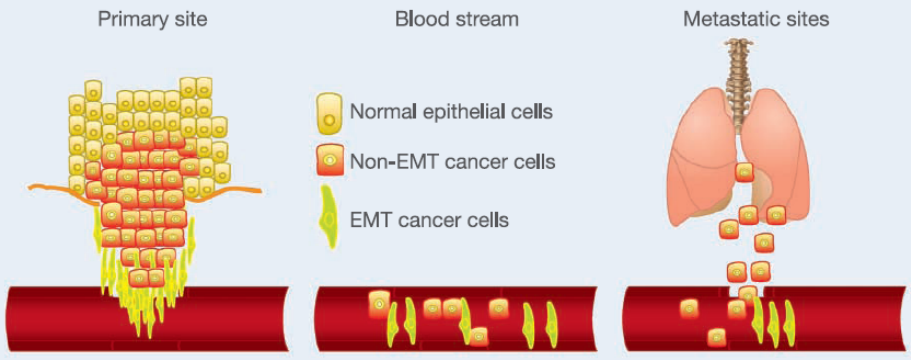

Figure 13 | The cooperation theory. According to the cooperation model, the primary tumor consists of a mixture of EMT and non-EMT cells. The EMT cells lead the way for the non-migratory non-EMT cells towards and into the local vasculature. Once in the bloodstream, only the non-EMT cells are able to extravasate and establish metastasis, in this case in the lungs (Adapted from Tsuji et al., 2009).

easily, possibly also due to cell-cell adhesion. Although the authors tested a 5 times higher number of EMT cells, still no metastasis were formed. The absence of EMT-cell extravasation could also be caused by the fact that these are induced EMT cells via Twist2, and other important molecules, i.e. in regulating CAMs to adhere to the endothelium or cytoskeletal reorganizers to squeeze between vascular endothelial cells, are under the regulation of one or more of the other EMT-inducing transcription factors. In fact, analysis showed that no Snail1 and Snail2 was expressed in the cells, which have both shown to be important regulators of many EMT-processes (Tsuji et al., 2008). In mice lacking Snail during embryogenesis, for example, cells cannot undergo a full EMT and the cells maintain their original epithelial phenotype (Carver et al., 2001). Thus, metastasis could also rely on a combination of cells undergoing EMT and cells remaining in the epithelial cell state. To be more conclusive however, the induction of EMT in these experiments should more reflect the total pattern of transcription factors described in chapter 1 to 4, EMT is not solely induced by expression of Twist2.

Furthermore, these observations support the idea that the cells that actually form the metastasis remain E-cadherin positive during the whole metastatic cascade and rules out MET (Wells et al., 2008). To test this possibility, the authors tested EMT cells that re-expressed E-cadherin, and found that these were unable to promote metastasis after intravenous injections (Tsuji et al., 2008). This could mean that MET has no significant role during metastasis, or that this experimental setup does not resemble the endogenous MET process and thus more research is required to be conclusive. Indeed, in order to be completely convinced of the cooperation theory, individual cells have to be tracked and their phenotype observed all through the course of the metastatic spread *in vivo* (Micalizzi et al., 2010). Otherwise, it could be possible that some cells did undergo spontaneous MET by extracellular stimuli, and thus that EMT cells, after an MET, are part of the new colony forming tumor mass.

Another possibility could be that cancer cells do not undergo a full, but rather a partial EMT. These cells were also shown not to migrate as singular, mesenchymal cells, but as a multicellular aggregate of cells, a process known as collective migration (Friedl and Wolf, 2003). Collective migration is not solely observed in tumor cell migration, but, as with many mechanisms seen in carcinogenesis, also seen during embryonic development (Cooper and D'Amico, 1996). However, cells that migrate as a cluster are less malignant compared to fully mesenchymal transitioned epithelial cells, and only gain a few of the mesenchymal characteristics of cells undergoing complete EMT, to increase the invasiveness. In an experiment using MDCK cell xenografts in mice, for example, the induced expression of an MMP, MT1-MMP, was enough to initiate invasiveness. The cells formed a tubular single layer surrounding a lumen and were polarized. As such, the cells could enter both lymphatic and blood vessels, but no metastases were detected using this technique, possibly due to the fact that the expression of one MMP is not sufficient to complete all the steps of metastasis (Soulie et al., 2005).

Next to collective migration, incomplete EMT can also result in single-cell migration. This mode of migration is more malignant compared to clustered migration, since the tumor cells also lose the expression of certain CAMs, next to gaining invasiveness. As described above, the induction of EMT involves many different extracellular signals, affecting multiple intracellular signal transduction pathways. The presence of the molecules involved in these pathways differ between cell types, so the way different cell types react on a single signal from the microenvironment can differ (Christiansen and Rajasekaran, 2006). In an experiment to test TGFβ-

induced EMT, 20 different human and mouse cell lines and cultures of primary epithelial cells were tested on occurrence of EMT, both molecularly and by morphology. The authors found that only 2 out of the 20 lines underwent EMT (Brown et al., 2004). Thus, one signal can lead to EMT in one cell type, but have no effect in the other. These experiments were performed *in vitro*, where other variables were kept constant. *In vivo* however, the differential reaction is probably even bigger, due to the amount of signals from the microenvironment that all have the potential to effect one or more of the molecules important during EMT.

In conclusion, there are multiple models which indicate the role of EMT during carcinogenesis. Cells can undergo full EMT followed by MET, or partial EMT, with multiple phenotypical outcomes. EMT and non-EMT cells were even shown to cooperate in some models. However, as stated in the beginning of this discussion, some researchers do not believe there is proficient evidence for the role of EMT during carcinogenesis *in vivo* and rather make a difference between the true developmental EMT and describe the cellular conversions observed in cancer progression as an EMT-like phenotype (Klymkowsky and Savagner, 2009). The markers used to show EMT namely, are not specific for mesenchymal cells that have undergone a transition from epithelial cells. Furthermore, there is little evidence for EMT *in vivo*, for that reason Klymkowsky *et al.* prefer the term EMT-like. The processes that occur during EMT, can namely also be due to the increased genomic instability and consequent genetic alterations, that give the cells the hallmarks to become metastatic (Hanahan and Weinberg, 2000). Thus, EMT-like implies a state in which the metastatic characteristics are gained as single steps and not as the single, conserved EMT where a radical change from epithelial to mesenchymal cell states takes place (Klymkowsky and Savagner, 2009). According to the 'EMT-like theory', no dedifferentiation is required and the observed more mesenchymal and invasive phenotypes result from tumor cell renewal and adaptation to the changing microenvironment, i.e. hypoxia and the secretion of growth factors.

Moreover, some reports not only question the role of EMT in carcinogenesis, but also during physiological processes as wound healing (for review see Tarin et al., 2005). And indeed, besides the presented models of EMT and EMT-like processes, cells have also shown to metastasize using completely different mechanisms. For one, losing cell-cell adhesion alone is not EMT. Besides the key function of E-cadherin according the EMT theory, E-cadherin has also shown to play an important role in non-EMT related metastasis. In a mouse tumor model with an epithelial-specific knockout of p53, deletion of E-cadherin resulted in the development of invasive and metastatic mammary carcinomas (Derksen et al., 2006). Even more interestingly are experiments looking at the role of the small mucin-like protein podoplanin, which is extensively found in cell surface protrusions. Podoplanin was shown to be upregulated in several human cancers, suggesting a role in tumor progression. Subsequent *in vitro* and *in vivo* data showed that podoplanin induced tumor cell invasion and increased malignancy in MCF7 breast carcinoma cells. The increased malignant phenotype was not due to EMT, since epithelial markers, i.e. E-cadherin, were unaltered by podoplanin. Furthermore, no upregulation of any mesenchymal markers was detected, i.e. N-cadherin. Podoplanin, however, was shown to induce collective migration by downregulating Rho GTPases, one of which was RhoA (Wicki et al., 2006). In contrast, others have found that in MDCK cells, podoplanin induced EMT including the loss of E-cadherin, most interestingly through activation of RhoA (Martin-Villar et al., 2006). Again this shows how different stimuli in different cell types lead to altered cellular outcomes, possibly due to differences in basal levels of RhoA between both cell types and the cellular effects of RhoA activation.

Besides the earlier mentioned mechanisms of migration, cancer cells can also move as leukocytes. Leukocytes can rapidly move through the ECM, by quickly changing cell shape and squeeze themselves through the matrix, rather than degrading it (Figure 14). This mode of cancer cell movement, termed amoeboid, is observed using intravital imaging, where cancer cells adopt leukocyte-like morphologies (Wyckoff et al., 2000). This movement, like the earlier described migration methods, requires reorganization of the actin cytoskeleton (Madsen and Sahai, 2010). Because this type of movement relies primarily on rapidly changing cellular morphology, MMP activity is not required, suggesting the ECM has large enough gaps for cellular migration. Even more, even the crossing of BMs by cancer cells is seen in the absence of histologically detectable degradation of the membrane. Furthermore, also unlike migration during EMT, integrin dependence is limited (Madsen and Sahai, 2010).

Summarizing, although metastasis is the main cause of death during cancer, the exact cellular mechanisms that lead to the different steps of metastasis are still poorly understood (Weinberg, 2008). EMT seems provide an answer for some of the questions, however the transition itself is also not completely unraveled yet, let alone the EMT-independent processes that occur in cancer cells and also lead to a metastatic phenotype. The biggest problem is that individual cells could not be followed in their natural microenvironment. Recent advances in microscopy now allow us to follow selected tumor cells *in vivo*, for long periods of time. For instance, using tumor cells expressing photoswitchable fluorescent proteins in combination with a Mammary Imaging Window (MIW), cells anywhere in the tumor can be marked and tracked over time through the MIW in living mice (Kedrin et al., 2008). These intravital imaging techniques will give us the answer to several vital questions concerning the process of metastasis. It can be shown whether single cells indeed undergo EMT, followed by MET, or if tumor cells only undergo a partial transition towards a mesenchymal phenotype. Furthermore, these techniques may prove if EMT is a single conserved event or that all the steps of metastasis are singular lose events, i.e. gained due to genetic alterations caused by genomic instability.

Increasing our insight into the role of the transcription factors that have shown to play a role during EMT, and pinpointing their exact function in the process and which molecules they regulate, will greatly enhance our knowledge of the metastatic process. This knowledge will subsequently contribute to better prognosis and treatment of cancer.

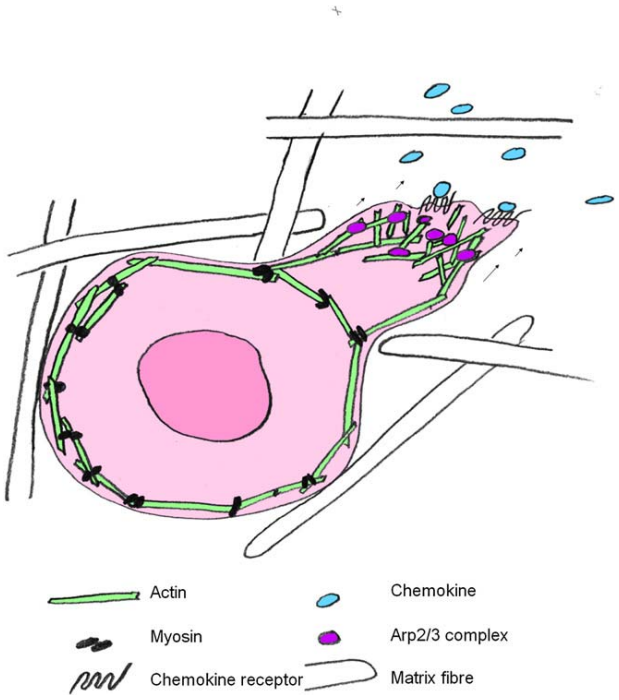

Figure 14 | Amoeboid movement of cancer cells. Next to the MMP and integrin dependant migratory processes cells use during EMT, cancer cells can also migrate in an amoeboid fashion. This leukocyte-resembling mode of motility requires rapid actin reorganization, after with the cells can squeeze themselves through gaps in the interstitial tissue (Madsen and Sahai, 2010).

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