Invest Clin 54(2): 186 - 205, 2013

Epithelial-mesenchymal transition (EMT): principles and clinical impact in cancer therapy.

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Keywords: differentiation, cell polarity, cadherins, cancer, microRNA, cancer stem cells.

Abstract. The epithelial-mesenchymal transition (EMT) is a biological phenomenon responsible for the formation of different tissues and organs during normal metazoan development. Because of the connection of the EMT with the pathogenesis of certain diseases, such as cancer, the attention of the scientific community has been directed towards the search for and identification of effective therapeutic targets. These targets include signal transduction in cancerous stem cells and the use of microRNAs, which would inhibit EMT-associated phenotypic changes and tumoral progression. In an attempt to compile relevant and current information, this work addresses concepts that define the EMT and the advances in this field. The wealth of knowledge gained from areas such as the loss of cell polarity and intracellular adhesion complexes, the signaling pathways implicated, microRNA participation in this process, and stemness acquisition in embryonic and cancerous cells, all of which allow for the visualization of promising perspectives, particularly, methods for targeting advanced malignancies, are presented herein.

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Transición epitelio-mesenquimática (TEM): principios e impacto clínico en la terapia contra el cáncer.

Invest Clin 2013; 54(2): 186 - 205

Palabras clave: diferenciación, polaridad celular, cadherinas, cáncer, microARNs, células madre cancerosas.

Resumen. La transición epitelio-mesenquimática (TEM) es el fenómeno biológico responsable de la formación de los diferentes tejidos y órganos durante el desarrollo normal de los organismos metazoarios. En razón de su conexión con la patogénesis de ciertas enfermedades como el cáncer, la atención de la comunidad científica se ha redireccionado hacia la búsqueda e identificación de blancos terapéuticos efectivos, como la transducción de señales de las células madre cancerosas o la utilización de microARNs, que permitirían bloquear los cambios fenotípicos asociados con la TEM y, por ende, la progresión tumoral. En un intento por recopilar información relevante y actualizada, el presente trabajo aborda conceptos que definen a la TEM y avances alcanzados en este campo. El acervo de conocimiento obtenido en aspectos como pérdida de la polaridad celular y de los complejos de adhesión intercelular, vías de señalización implicadas y participación de los microARNs en el proceso, así como adquisición de stemness o troncalidad, tanto en células embrionarias como cancerosas, hace posible visualizar perspectivas promisorias, en especial en lo que se refiere a las terapias contra las malignidades de alto grado.

Received: 24-09-2012. Accepted: 13-12-2012

INTRODUCTION

The epithelial-mesenchymal transition (EMT) is an evolutionary strategy. Over 600 million years ago, the EMT made possible the generation of mesenchymal tissue from epithelia, which on turn made possible the tissues and organs that constitute metazoan organisms (1). The EMT has recently become an attractive research target because the loss of epithelial cell polarity and the manifestation of characteristics associated with the new mesenchymal phenotype are crucial events, not only of normal embryogenesis and developmental processes, but also of pathologic stress situations, such as the fibrosis associated with tissue degeneration, regeneration and cancer (2). These data explain why, despite being discovered over three decades ago, over 50% of EMT papers have been published in the 2010-2012 period, according to information obtained from databases such as the ISI Web of Knowledge and the US National Library of Medicine.

Although the coexistence of epithelial and mesenchymal cells in the same tumor mass was reported in 1978 (3), which suggested a type of phenotypic transformation, it was not until 1995 that the EMT was characterized as the biological process associated with the appearance of epithelial cell morphology and cytoskeleton modifications. These cells develop the capacity to respond to specific signals from the neighboring extracellular matrix (4). It has been

demonstrated that during this process and in response to an extrinsic signaling mechanism that is not fully understood, these cells undergo a series of transformations, including the loss of adhesion complexes and the polarity axis (with decreased epithelial markers such as E-cadherin, desmoplakin, and plakoglobin), hypertrophy and cytoskeleton reorganization (with the substitution of keratin filaments for vimentin). Other changes include increased mesenchymal marker expression (N-cadherin, smooth muscle α-actin, fibroblast specific protein 1, fibronectin, and collagens I and III) and increased matrix metalloproteinase activity (MMP-2, -3, and -9). These changes are associated with the acquisition of migratory behavior and invasiveness (4-8).

The EMT is critical for the normal course of vertebrate and invertebrate embryonic development; it enables the appearance of the mesodermic layer in the germinal disc or the formation of the neural tube (9). The EMT can occur in a physiological context and in diverse pathological settings. This fact determines which particular EMT characteristics exhibit variations, depending on the different environments that transitioning cells confront. Thus, inflammation is associated with the EMT program that occurs during tissue regeneration, chronic degeneration, mature organ fibrosis and tumor development but not during embryogenesis; blood and lymphatic dissemination are exclusive of the EMT program observed in tumor development (10).

Cells that have experienced the EMT retain their plasticity properties, which make reversion to an epithelial phenotype possible (known as the mesenchymal-epithelial transition, MET). Thus, the formation of most adult tissues requires one or more rounds of EMT and MET, as observed in the normal development of organs such as the kidney, in which the excretory tubules are formed when mesenchymal cells adjacent to the collecting system tubules differentiate into epithelium. The expression of adhesion molecules, such as E-cadherin and the secretion of basal lamina components, such as syndecans and laminin (11), mediates this event. This process is directed by the genes PAX2 (Paired box 2), BMP-7 (Bone morphogenetic protein 7) (12), and WT1 (Wilms tumor) (13).

Additionally, the cellular mechanisms that initiate the EMT during embryogenesis and development can also be activated in carcinogenesis (14, 15). It is only in carcinogenesis, and not under other conditions, that these mechanisms allow epithelial neoplastic cells to experience most of the stages of the invasion-metastasis cascade, including the acquisition of motility and invasiveness, in a process that is associated with a high malignancy degree, which can occur early or late during tumor development (16, 17). However, MET is involved in the final phase of metastasis. After extravasation and migration to anatomical locations different from their origin site, cancer cells reacquire the epithelial phenotype necessary for the formation of secondary tumors as a response to the encounter of microenvironments that lack signals found in the primary tumor (9).

After undergoing the EMT, some cancer cells may express phenotypes characteristic of stemness (18). However, it is possible that the EMT is not sufficient for developing this feature *per se*; taking into account that stemness has been considered indispensable for cells to adapt to the new and strange microenvironments of distant tissues (19). In addition, phenomena such as the resistance to apoptosis mediated by the loss of natural attachment to epithelial tissue (anoikis) (20, 21) or the acquisition of chemoresistance (22) have also been associated with the EMT.

Considering that the keys to unlocking the cellular and molecular mechanisms that mediate tumoral transformation and metastatic foci formation can be found in the EMT observed in embryonic development (2, 15), the scientific community has moved its focus of interest in that direction, with the objective to identify previously elusive clues that will allow for the early detection of cancer or the identification of effective targets for cancer treatment.

CELL POLARITY AND ADHESION COMPLEXES

In mammals, epithelial polarity is determined by the asymmetric distribution of certain proteins in specific domains of the plasma membrane. The organization of the normal epithelium continuum, in which cellular cohesion is ensured by tight or occlusive junctions that seal the apical intercellular space, originates in two compartments: the apical and the basolateral. Under the *zonula occludens* and forming an axis in an apical-basal direction are three types of adherence junctions: *zonula adherens,* also known as belt desmosomes; *macula adherens,* or punctate desmosomes; and hemidesmosomes. Of these, the first two belong to the adherens junctions, where the adhesion molecules cadherin and catenin are present.

Unlike adherens junctions, hemidesmosomes affix the basal plasma membrane to the tissue's basal membrane (cell-extracellular matrix junction) via integrins $\alpha_6\beta_4$ and laminin 5 filaments. These molecules allow the anchoring of the cytoskeleton keratin filaments to the plasma membrane and to the basal lamina.

All the intercellular junctions described are responsible for ensuring epithelial tissue stability, which means that for the EMT to initiate, the epithelia must lose the cell-cell and cell-basal lamina junction complexes (23) and, thus, their polar architecture. In addition, the epithelial basal lamina must disappear, exposing epithelial cells to extracellular matrix adhesion points or to signaling molecules produced by the degradation of the basal lamina (24).

The establishment of apical-basal polarity depends on the cooperation of proteins such as Crb3 (Crumbs homolog 3), hDlg (human Discs large), Par3/Par6, and the cadherins, among others. Crb3 is an apical transmembrane protein whose expression negatively regulates the TGF-β (Transforming growth factor β) signaling pathway, a potent EMT inducer, and the tumor suppressor pathway Hippo (25). hDlg, a cytoplasmic protein of the MAGUK (Membrane-associated guanylate kinases) homologue family, is localized adjacent to intercellular junctions, where its interaction with E-cadherin in confluent epithelial cells has been demonstrated. This tumor suppressor protein has been shown to have roles in actin cytoskeleton regulation and in the stabilization of adherens junctions (26, 27). In addition, the Par3/Par6 complex reduces ROCK (Rho-associated kinase)-mediated actomyosin contraction in intercellular junctions, an important cytoskeleton regulator. Therefore, when cadherins or the Crb3, hDlg, Par3/Par6 genes are suppressed, the polarization pattern disappears (28), promoting cell migration, particularly collective cell migration (29).

Cadherin-catenin system

Background. Because their extracellular sites have an affinity for calcium, cadherins (Calcium-dependent adhesion) play a central role in cell polarization and adhesion (30). They also participate in cell segregation processes, morphogenesis, and the preservation of epithelium integrity (31). Initially described in 1981 by Hyafil et

al. (32), these transmembrane proteins have been grouped into four families according to the number of calcium binding site repetitions. The most studied family is type I, or classic, to which E-cadherin belongs, and this protein family is found in most epithelia (33); N-cadherin is found in neural, striated, and skeletal cardiac muscle and in mesothelial and mesenchymal tissue (34, 35). In addition, P-cadherin is found in the placenta (36), VE-cadherin is found in the vascular endothelium, and R-and K-cadherin are found in the retina and kidney, respectively (38).

In adherens junctions (Fig. 1), cadherins form plates or cytoplasmic condensations that are localized near the lateral plasma membranes of adjacent cells. From there, their glycosylated residues project towards the extracellular space, overlapping and forming homodimers in a calcium-dependent manner. Inside the plates, β-catenin and p120-catenin proteins are bound to provide support to the cytoplasmic domains of the classic cadherins and to α-catenin (39). Binding these proteins enables the formation of complexes that are, in turn, connected with actin filaments (in the case of *zonula adherens* junctions) or with keratin filaments (in the case of *macula adherens* junctions).

Main alterations

The cadherin-catenin system is susceptible to modifications such as the loss of adhesive and polarity properties. This condition can initiate the EMT, facilitating the acquisition of the migratory behavior required by epithelial cells to move to sites far from their tissue of origin. This effect occurs in both normal embryonic development (40) and tumor progression (41).

E-cadherin loss

The loss of E-cadherin is one of the most remarkable characteristics of all the EMT types, resulting in the disintegration of adherens junctions and the disappearance of cell polarity. In embryogenesis and development, E-cadherin loss results from the activation of FGF/FGFR1 signaling (Fibroblast growth factor/Fibroblast growth factor receptor). This activation then leads to the activation of EMT-associated tran-

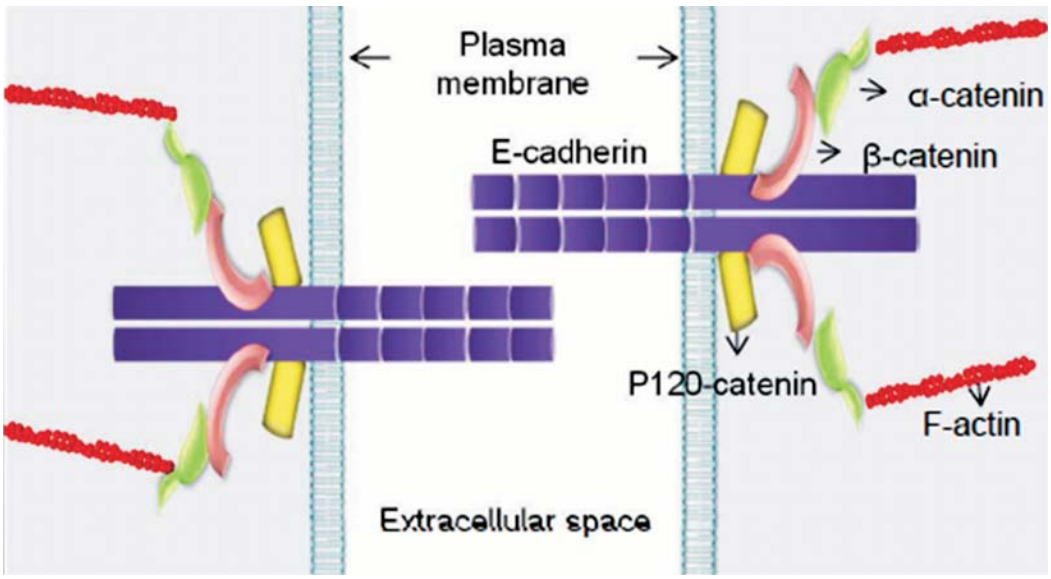

Fig. 1. Schematic representation of adherens junctions and of the interaction between the cadherin-catenin system and the cellular cytoskeleton.

scription factors (EMT-TFs), including Snail1 (42, 43), MAPK (p38 mitogen-activated protein kinase) (44), or EPB41L5 (Erythrocyte membrane protein band 4.1 like 5), which inhibits the p120-catenin-E-cadherin association (45). However, evidence has shown that the E-cadherin gene is negatively regulated by an EMT-TFs group that includes Snail1, Snail2/Slug, Twist, and Zeb2/Sip1/ZFXH1B in embryonic and tumor cells (46-52). In cancer cells, the loss of E-cadherin is considered a poor prognosis factor because there is a proportionally inverse relation between its level of expression and the degree and stage of the tumor (53). In addition to the activity of transcriptional repressors induced by growth factors (54), other factors can mediate the functional loss of E-cadherin, such as hyper-methylation of the gene's promoter region, which has a potent inhibitory effect (55), and to a minor extent, the enzymatic cleavage of the protein (56), changes in chromatin structure (57), or rare mutations in the germinal or somatic lines (58). Other factors that negatively regulate E-cadherin have been recently described, such as the microRNA miR-9 (59) or the oncogenic protein metadherin (MTDH) (60). These events promote migratory and invasive behavior in malignant cells via induction of the EMT. It has been demonstrated that the overexpression of MTDH in diverse types of cancer cells, such as breast cancer cells, can produce not only a reduction of E-cadherin expression but can also result in up-regulation of EMT-TFs, such as Snail1 and Snail2/Slug, and of the mesenchymal markers fibronectin and vimentin in an NF-κβ-independent manner (60).

E-cadherin/N-cadherin switch

The EMT is usually accompanied by E-cadherin loss and N-cadherin up-regulation. Compared with epithelial cells, this change causes a reduction in mesenchymal cell polarization, inducing the cells to acquire motility properties through mechanisms that are not completely understood, and allows the cadherin switch to associate with the invasive phenotype of cells transformed by the EMT (61). However, the term "switch" does not refer exclusively to changes in the mRNA levels of both genes but also refers to the co-expression of E-cadherin with N-cadherin or other cadherins (P, T, R, and 11) (62).

The participation of N-cadherin is crucial for key events of embryonic development, such as the growth of the neural plate, the migration of cells originating from the neural crest, mesoderm formation during gastrulation, somitogenesis, and heart tube formation (63, 64). N-cadherin is also crucial for tissue regeneration, promoting the remodeling of the connective tissue, and wound healing (65).

In most malignant tumors, E-cadherin repression is followed by the induction of N-cadherin expression. This step leads to the development of an especially aggressive behavior that is characterized by the acquisition of migratory capacity and the reorganization of the actin cytoskeleton that is concurrent with increased proliferation. The interaction of N-cadherin with PDGFR (Platelet-derived growth factor) has been associated with these events (66). It has been demonstrated that N-cadherin can also interact with FGFR, which results in the activation of the corresponding signaling pathway and in the inhibition of the internalization of the complex formed by FGF and its receptor (67), thus leading to sustained MAPK activation and increasing invasiveness, migration, and MMP secretion. In addition, N-cadherin can increase apoptosis resistance in tumor cells via positive FGFR regulation (68). It is within this context that the loss of E-cadherin expression has been associated with advanced tu-

mor stages and poor prognosis (53), whereas the up-regulation of N-cadherin has been related to motility and migration induction and cancer cell metastasis (62, 68).

Similar to other classic cadherins, N-cadherin consists of an N-terminal extracellular domain with homophilic sites that interact with their homologues in adjacent cells and a cytoplasmic domain that interacts with β-catenin, allowing N-cadherin to interact with the cytoskeleton, thus ensuring the stability of the adherens junctions in which it participates. The E-cadherin/N-cadherin switch has been demonstrated in several types of carcinomas, in which E-cadherin loss as a poor prognosis factor and a marker of advanced tumor stages contrasts with increased N-cadherin expression, which is indicative of enhanced motility and invasiveness. Considering these data, how can we solve the contradiction posed by an increase of N-cadherin leading to the induction of motility and invasiveness with the promotion of cell adhesion? In this regard, and with the objective of explaining the switch of E-cadherin for N-cadherin, the current classic dogma proposes that after the disappearance of intercellular adherens junctions, which resulted from E-cadherin loss, the affected cells move from the epithelial stratum and reach subjacent tissues while N-cadherin expression not only facilitated adhesion to other N-cadherin-positive cells, such as endothelium or stroma connective tissue, but was also sufficient for the initiation of malignancy (67, 69). In a surprising turn, Maret *et al.* (64) demonstrated the expression of the non-adhesive precursor pro-N-cadherin in the plasma membranes of cells from different types of tumors and how this expression is related to the development of migratory and invasive characteristics. Thus, in premalignant lesions, E-cadherin loss was followed by the expression of N-cadherin and pro-N-cadherin, whereas in malignant cells derived from high-grade tumors and their metastases, pro-N-cadherin levels were higher than the levels in low-grade tumors. Because of the E-cadherin/N-cadherin-pro-N-cadherin switch, the affected cells partially retained their adhesive characteristics. This effect could be due to the possible interference of pro-N-cadherin with N-cadherin homodimerization, leading these researchers to propose that, more than the type of cadherins expressed, it is the relative differences in the adhesion strength that determine the invasiveness of cancer cells. In addition, Elmoneim and Zaghloul (70) reported a significant increase of breast cancer cell motility associated with N-cadherin and E-cadherin co-expression. This result demonstrates that the loss of E-cadherin or expression of N-cadherin alone is insufficient to promote migratory and invasive behavior.

β-catenin translocation

Another protein found in adherens junctions is β-catenin, which is responsible for the transmission of the contact inhibition signaling that inhibits epithelial cell proliferation. In addition to forming part of the cadherin-catenin adhesion complexes during embryonic development and tumor transformation (71), this protein participates in the Wnt signaling pathway. Thus, when it is not part of cadherin complexes, β-catenin accumulates in the cytoplasm, where it is phosphorylated by GSK-3β (glycogen synthase kinase 3β), which is part of the APC/axin/ GSK-3β complex. This phosphorylation is followed by ubiquitination and proteasomal degradation (72). Instead of phosphorylation and subsequent degradation, β-catenin can translocate into the nucleus, where it acts as a TCF-1 (T-cell factor-1) or LEF-1 (Lymphoid enhancer factor-1) cofactor and activates the transcrip-

tion of genes implicated in cell proliferation, such as c-Myc and the D1 and D2 cyclins. β-catenin also contributes to the activation of genes involved in the acquisition of migratory and invasive capabilities, such as Snail2/Slug, MMP7, vimentin and fibronectin (74, 75). The β-catenin-LEF-1 complex can also repress E-cadherin transcription via binding to the promoter region of the gene (76).

p120-catenin translocation

When interacting with EPB41L5, β-catenin induces the disintegration of the cadherin-catenin complexes (45), moves to the cytoplasm, and accumulates, which leads to the instability of the intercellular adhesion complexes. In addition, β-catenin promotes the formation of invadopodia (lamellipodia and filopodia), which are required for the acquisition of migratory capabilities. These effects result from p120 regulation of signal transduction associated with the actin cytoskeleton dynamics. p120 represses the activity of small GTPases of the Rho family (77), such as RhoA, to stabilize adhesion complexes. Other examples of the small GTPases are Rac and Cdc42, which participate in lamellipodia and filopodia formation, respectively. At the nuclear level, the binding of p120-catenin to Kaiso, a transcription factor of the BTB/POZ-ZF type (BTB, broad complex, Tramtrack, Bric á brac; POZ, poxvirus and zinc finger; ZF, zinc finger), does not affect Wnt signaling. However, this binding can ameliorate the Kaiso-mediated repression of RhoA-ROCK signaling activation. It can also promote microtubule destabilization and inhibit the tumor suppressor pathway Hippo (78, 79).

Although the effect of p120-catenin on proliferation or on the cell cycle in general is not completely known, Jiang *et al.* (80) demonstrated that the complex formed by isoform 3 of p120-catenin and Kaiso in the nucleus derepressescyclin D1. This event is mediated by the exportation of the transcription factor to the cytoplasm, where isoform 1 stabilizes β-catenin, which increases β-catenin activity.

SIGNALING

The EMT program begins as result of the activation of complex signaling pathways that can be induced by soluble growth factors, including its most powerful inducers, the members of the TGF-β superfamily, FGF (Fibroblast growth factor), Hedgehog, and Wnt proteins. The EMT can also be initiated by components of the extracellular matrix, such as collagen or hyaluronic acid (10). Signaling effectors include the small GTPases Ras, Rho, and Rac or members of the Src tyrosine-kinase family, which promote the disintegration of adhesion complexes, cytoskeleton re-organization, and the activation or repression of EMT-TFs (1). This group of nuclear signaling targets associated with the EMT includes Snail homologous zinc finger transcription factors (Snail1, Snail2/Slug, and Snail3) and other types of helix-loop-helix-containing proteins, such as Twist/Twist1, Zeb1/TCF8/δEF1, Zeb2/SIP1/ZFXH1B, and TCF1/E47/E12, all of which are E-cadherin repressors (8,81-84).

During embryonic development

It has been demonstrated that in the bilaminar germinal disc signaling initiated by Wnt and members of the TGF-β superfamily, such as Nodal and Vg1, are necessary for gastrulation to occur in the EMT context. FGF and its receptor cooperate in this event with the activation of EMT-TFs Snail1, Eomes and Mesp (2, 85). Notch signaling has also been implicated in the up-regulation of Snail1 and Snail2/Slug, in the reduction of epithelial marker expression, and in the increase of mesenchymal

markers (86-88). Through these methods, Notch signaling regulates stem cell proliferation and histogenesis and controls differentiation, cell division, and apoptosis in adult tissues (89). Other signaling pathway mediators associated with actin cytoskeleton dynamics are regulated by cadherins, such as PI3K (Phosphatidylinositol 3-kinase) or EGFR (Epidermal growth factor receptor). This observation highlights the complexity of the interactions among molecules that comprise adhesion complexes and of the signal transduction responsible for cytoskeleton reorganization, and, therefore, the control of cellular shape and behavior during embryonic development (31, 90).

In the pathogenesis of fibrosis in mature organs

In fibrosis, excessive amounts of collagen secreted by interstitial fibroblasts that have been transformed into myofibroblasts by the EMT facilitate the formation of a fibrotic net that diminishes the functionality of the affected tissue. In addition, terminally differentiated cells, such as the epithelium from the excretory and collector kidney tubules, cornea, lung alveoli, hepatocytes, cardiomyocytes and endothelial cells, can undergo the EMT and contribute to fibrosis (1). TGF-β signaling is crucial for this type of EMT and is involved in the activation of the transcription factors Snail1 and Zeb1/TCF8/δEF1 (7). Because of this activation, the transcription of genes involved in the establishment of polarity is repressed, which leads to subsequent alterations of the actin cytoskeleton and in the loss of the polarity axis (28, 91, 92). NF-κβ is also involved in fibrotic disorders. It has been demonstrated that via can induce Snail1 transcription and stabilization and the loss of E-cadherin expression, thus initiating the EMT and fibrosis of mesothelial tissue subjected to peritoneal dialysis (93).

In malignant progression

In primary cancer cells, EMT-associated signaling is initiated via heterotypic interactions with adjacent tumor stromal cells. Thus, extrinsic growth factors secreted by stromal cells can successfully act on cancer cells that have developed an adequate response capacity, resulting from genetic and epigenetic transformations undergone during malignant transformation progression (2) (Fig. 2). In particular, the role TGF-β plays in inducing gene and protein expression has important pleiotropic connotations, considering the dual role of TGF-β in tumor progression. On the one hand, TGF-β acts as a suppressor during early stages, inhibiting proliferation and inducing apoptosis, while on the other hand, TGF-β promotes tumorigenesis and the invasion-metastasis cascade in advanced stages (94, 95). Regarding the pro-tumorigenic effect, the EMT acts as a convergence factor for different TGF-β-activated signaling pathways, which activate different effectors leading to the development of characteristics that favor the invasion-metastasis cascade. Thus, PI3K/AKT activation is essential to promote resistance to cellular damage and death (96), whereas NF-κβ inhibits apoptosis, therefore facilitating tumor growth (97, 98). RhoA promotes the capacity in cancer cells to migrate (99), whereas ILK (Integrin-linked kinase) and MAPK (ERK1/2, JNK and p38) reduce the expression of epithelial markers, such as E-cadherin, and increase mesenchymal markers, such as fibronectin (100). According to Tomlinson *et al.* (101), FGFR1 activation is required for MAPK to induce the capacity to migrate and invasiveness, which are phenotypes that can be potentiated by the concomitant activation of PLCγ (Phospholipase C gamma). Boudreau *et al.* (102) reported that TGF-β can induce –in a SMAD3-dependent manner– the expression of Nox4, a member of the NADPH oxidase

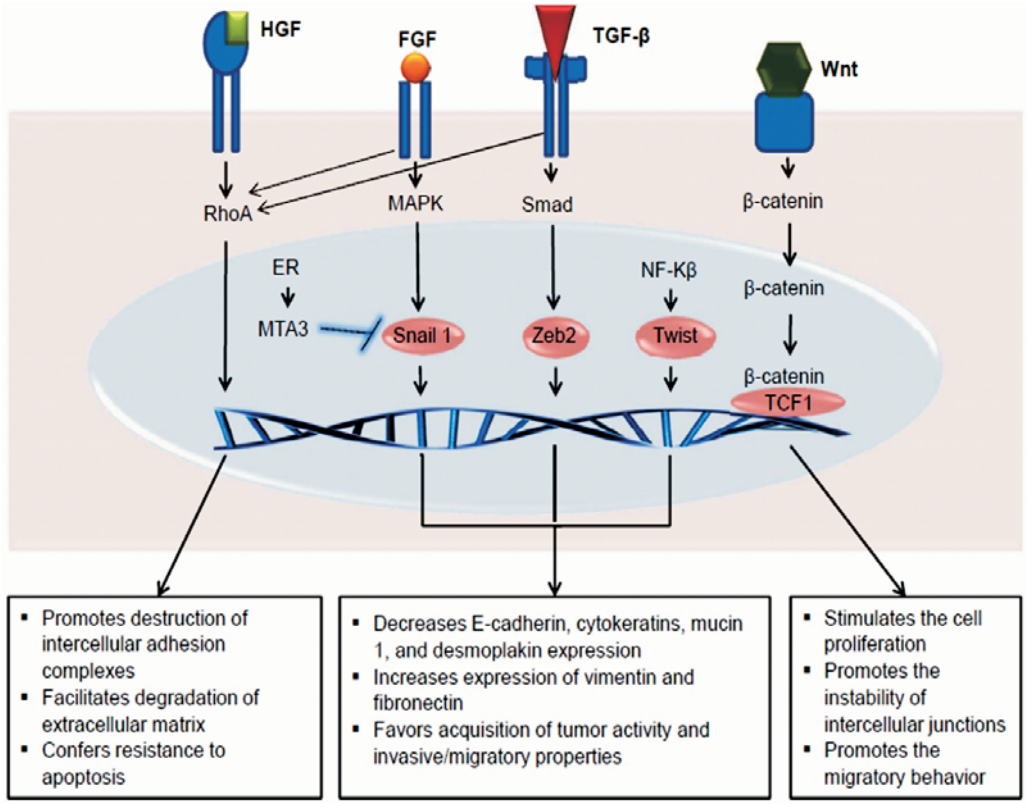

Fig. 2. Main signaling pathways associated with the EMT during malignant progression.

family, at the mRNA and protein levels, and ROS production. These events induce migratory behavior and increase the expression of fibronectin in normal and metastatic mammary epithelial cells.

Interaction amongst cancer cells and the tumor microenvironment. Because of the activation of signaling associated with the EMT in cancer cells, EMT-TFs are induced or activated, which results in the expression of malignant phenotypes. In addition, the same cancer cells can secrete signals that stimulate the production of inflammatory mediators by mesenchymal stem cells of the neoplastic stroma. These mediators act on cancer cells to induce the expression of malignant phenotypes. Examples of these mediators include CCL5 (Chemokine [C-C motif] ligand 5), which promotes invasive behavior (103), TNF-α, and, to a minor extent, IL-1β, which induces effects such as E-cadherin repression, reduced β-catenin in the plasma membrane, vimentin expression, actin cytoskeleton reorganization, and increased adherence to substrate (104). Macrophages of the neoplastic environment also contribute to malignant progression via interaction with cancer cells –intratumoral cells and those in the invasive front– which induces the EMT through TGF-β and the activation of β-catenin (105).

There are also reports indicating the effect of hypoxia on the EMT. Thus, hypoxic microenvironments and the activation of the hypoxia signaling pathway HIF (Hypoxia-inducible factors) are factors that favor invasion and metastasis. These factors activate signaling pathways such as TGF-β, Notch, and NF-κβ, which induce the expression and activity of EMT-TFs such as Snail1, Snail2/Slug, Twist/Twist1, Zeb1/TCF8/δEF1, and Zeb2/Sip1/ZFXH1B (89).

CANCEROUS STEM CELLS

During the early stages of malignancy, the dynamics of the EMT program enables the initiation of an invasion-metastasis cascade after the rupture of the tumor mass. This process endows primary cancer cells to enter the nearest lymphatic and blood vessels, move through the vascular bed, and leave the vascular bed to establish small cancerous foci or micro-metastases in the parenchyma of distant organs. In these organs, angiogenesis can initiate and cause the formation of macroscopic metastatic foci. This stage has been named colonization (106) (Fig. 3).

It is worth noting that colonization is the only one of the stages in the cascade not associated with the EMT program, leading to the hypothesis that it is possible that cancer cells undergo additional modifications. The explanation of this process must consider that for the formation of macroscopic metastatic foci (macro-metastasis), it is necessary that micrometastases, the small groups of cells or even lone cancer cells that migrate to new microenvironments of organs or secondary tissues,

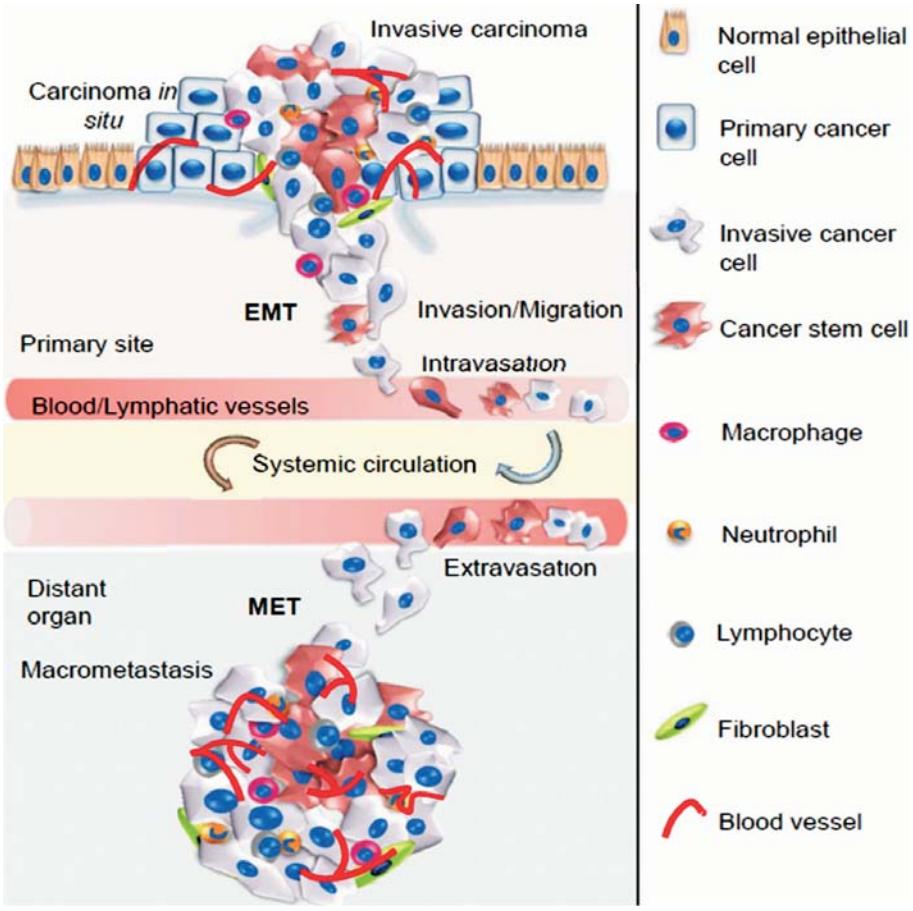

Fig. 3. The epithelial-mesenchymal transition (EMT) enables primary cancer cells to migrate and invade distant tissues. A large number of these cells develop stem cell characteristics, with the capacity to "seed" tumors and proliferate. Once the cells leave the blood and/or lymphatic stream, the cancer cells arrive in a new microenvironment, where they undergo a mesenchymal-epithelial transition (MET) and develop the capacity to colonize and generate secondary tumors or micro-metastases.

have developed the capacity to adapt and proliferate (17, 19).

Recent studies have revealed that the capacity to form new metastatic cell colonies (micro- and macro-metastases) resides in a small population of cells named tumor initiators or cancer stem cells (CSCs). This property has been demonstrated in breast, colon, brain, thyroid, prostate, head and neck, bone marrow, and other types of tumors (112). Pluripotency, the most notorious characteristic of CSCs, is largely responsible for the heterogeneity of the cell population present in the tumor mass. With a great capacity for self-renovation, CSCs act as new tumor seeds in the parenchyma of organs and secondary tissues reached during the colonization phase. This capacity is considered essential for cancer cells to establish a macro-metastasis. Therefore, completing the first stages of the metastatic cascade is not sufficient for cancer cells to acquire the capacities of self-renewal and proliferation (19). This finding begs the question: what is the critical factor for the expression of the CSC phenotype? It appears likely that the mechanisms associated with the EMT activation that induces normal epithelial cells to develop stemness characteristics also operate in cancer cells for them to develop the features of malignant stem cells (18, 113). In this way, the activation of the EMT in a primary carcinoma can generate a large population with motility and invasiveness characteristics and a smaller population of pluripotent CSCs with the capacity for limitless self-renovation and a high resistance to anoikis and chemotherapy (15, 18, 19, 21, 114).

Because of CSC differentiation, clones of different cells appear on secondary tumor masses. Some of these clones possess invasiveness characteristics, while others are composed of cells arrested in the cell cycle or with limited proliferative capabilities. Additionally, some of these clones are composed of self-renewing CSCs, which perpetuate the stemness phenotype. Even within the same tumor, CSCs can present different degrees of stemness, evidencing their notable phenotypic plasticity. This characteristic, combined with the amount of CSCs inside the tumor mass, acts as a determinant factor of metastatic growth aggressiveness (114).

METASTAMIR ROLE

There is a growing body of evidence regarding the role of microRNA (miRNA) in the post-transcriptional regulation of the EMT in both normal embryonic development and the invasion-metastasis cascade; these miRNAs have been given the name metastamiRs. These small, non-coding RNAs are endogenous and are part of the complex signaling pathways that include NF-κβ, EGFR, Twist/Twist1, Brms1, Zeb1/TCF8/δEF1, Zeb2/Sip1/ZFXH1B, and HIF-1α, which act as effectors of pro- or anti-metastatic signals (51, 115-117). By binding specific recognition elements located in the 3'UTR regions of the mRNAs of target genes (MRE, miRNA recognition elements), degradation or silencing of the miRNA may take place. Thus, it has been demonstrated that certain metastamiRs, such as the members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429), repress the EMT, and therefore, the development of cancer stem cell characteristics (118). This repression arises because of the miR-200-induced negative regulation of the EMT inducers Zeb1/TCF8/δEF1 and Zeb2/Sip1/ZFXH1B, which occurs through miR-200 binding to the coding mRNAs of these EMT inducers. This binding is part of a process that initiates the mesenchymal-epithelial transition (MET), thus inhibiting the EMT. Interestingly, it has been reported that in a dou-

ble-negative loop, Zeb1/TCF8/δEF1 promotes the transcriptional repression of the miR-200 genes when binding to their promoter regions (119).

The EMT repressor effect attributed to a miR-200 family member suggests that the possibility exists of using them as therapeutic targets against cancer, with the objective of EMT inhibition and malignant cell propagation. However, it has been determined that metastasis can also be promoted by miR-200 (120). This effect can be explained if we consider that they favor the MET that is required for the formation of metastatic foci. For this reason, the feasibility of using miR-200 in the early stages of tumor progression, when distant tissues have not been colonized and there are no micro-metastases, should be considered. Unlike the miR-200 repressor effect, other metastamiRs can favor the EMT. Such is the case for miR-9, which negatively regulates E-cadherin, thus promoting the activation of β-catenin signaling and increasing the expression of VEGF (Vascular endothelial growth factor) in breast cancer cells in vivo (59). The EMT is also favored by miR-103-107, which diminishes miR-200 levels in breast cancer cells, thus promoting an aggressive behavior and initiating the dissemination of these cells (121).

Thus, the functional role of metastamiRs has a dual effect on the EMT and the invasion-metastasis cascade because they can act as suppressors (let-7, miR-7, miR-16, miR-22, miR-31, miR-122, miR-126, miR-146a/b, miR-194, miR-200, miR-206, and miR-335) or as promoters (miR-9, miR-10a/b, miR-17-92, miR-21, miR-103-107, miR-214, miR-373, miR-378, and miR-520c) (122). This duality highlights the importance of miRNAs in the regulation of the transition between the epithelial and mesenchymal states and provides potentially useful therapeutic targets against cancer.

CONCLUSIONS AND PERSPECTIVES

Based on the advances obtained with the knowledge of cellular and molecular events involved in the EMT and its association with the metastatic cascade, it is possible to envision hopeful signs regarding the identification of new therapeutic targets against high-grade malignancies. In this sense, CSCs constitute a promising current subject of research, despite their resistance to most conventional chemotherapeutic agents. Singh *et al.* (123) recently reported that based on the capacity of CSCs to survive the deprivation of specific nutrients, their group was able to select rare cells with a mesenchymal phenotype similar to CSCs in highly aggressive breast cancer cell lines. This finding led them to propose that because of their highly adaptable metabolic state, these cells could be the primary cause of chemotherapy resistance. Consequently, these cells would be better targets for cancer therapy. However, the proposal by Singh *et al.* (123) did not consider that the effectiveness of anti-tumor strategies depends on inhibiting the expression of features associated not only with stemness but also with EMT, simultaneously eliminating two malignant cell subpopulations: CSCs and non-CSCs (124). In this regard, it is worth mentioning that the failure of therapies with angiogenesis or PARP-1 (Poly [ADP-Ribose] polymerase-1) inhibitors was due to their presenting a pro-EMT effect that induced stemness in non-CSC cancer cells. This point is more readily explained upon considering that anti-angiogenic agents induce hypoxia, whereas PARP-1 inhibitors weaken the inhibitory effect of PARP-1 on TGF-β signaling and stabilize Snail1 in malignant cells that have not previously expressed a stemness phenotype (125, 126). Within this framework, many efforts are currently directed at the identification of molecules that simultaneously in-

terfere with EMT-associated pathways in non-CSC tumor cells (such as TGF-β, PDGFR, and EGFR) and/or those pathways associated with the expression of stemness characteristics in CSCs (Wnt, Notch, and Shh).

Moreover, the strategy of silencing EMT inducers, such as Snail, with shRNAs has been shown to exert a double effect: it inhibits the epithelial-mesenchymal change and promotes the inverse mesenchymal-epithelial change via E-cadherin derepression (127).

In any case, it is clear that the design of effective therapies against cancer must consider evaluating the enormous cell heterogeneity of tumor masses, which results not only from the interaction of the tumor stroma with the cancer cells themselves, but from concurrent factors, such as the ischemic gradient generated within the tumor and the activation of exosomes that transfer genetic material among neighboring cells (114).

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