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Epithelial-Mesenchymal Transition in Metastatic Colorectal Cancer

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Abstract: Epithelial-mesenchymal transition (EMT) is a morphogenetic event during which cells lose their epithelial characteristics, such as apicobasal polarity, and gain mesenchymal features with an increased migratory and invasive potential. A wide range of studies have shown that this event plays a crucial role in tumor progression and metastasis. The results of the studies also demonstrate participation of EMT in therapy resistance and in the development and maintenance of stemness potential in colorectal cancer. In addition, evidence from preclinical and early clinical studies have shown that EMT markers might serve as outcome predictors and potential therapeutic targets in colorectal cancer. In this chapter, we discuss the fundamentals of EMT, including cell-cell adhesion disruption and cell polarity loss, actin cytoskeleton reorganization, transcription factors, and post-translational modifications associated with EMT. We also discuss EMT-mediated mechanisms of resistance to radiotherapy and chemotherapy. Finally, we provide a summary of EMT components and their use

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as potential markers or therapeutic targets for metastasis inhibition, along with the obstacles in the development of drugs targeting EMT.

Keywords: actin cytoskeleton reorganization; cell polarity loss; cell-cell adhesion disruption; epithelial-mesenchymal transition; metastatic colorectal cancer

INTRODUCTION

Colorectal cancer (CRC) is the third most diagnosed cancer (10%) and second most common cause of cancer-related deaths (9.4%) worldwide (1). Despite various advances in the screening, early detection, and management of established diseases, there is still a lack of innovative therapeutic treatments. Therefore, a herculean effort must be made to improve our understanding of this heterogeneous disease and develop an effective treatment to overcome this cancer by preventing metastasis to distant organs. Epithelial-mesenchymal transition (EMT), first described in embryogenesis, is a complex cellular process, in which cells transit between epithelial and mesenchymal phenotypes. Cancer is mainly characterized by the loss of apicobasal polarity and actin cytoskeleton disorganization. Consequently, epithelial features are lost in favor of mesenchymal features, increasing motility and invasiveness (2). Cells in this transition may also undergo the reverse process, Mesenchymal-Epithelial Transition (MET), which allows them to seed and form a secondary tumor (3).

Multiple signaling pathways, including TGF-β, Wnt, Notch, and FGF, can induce changes in epithelial cells by activating EMT-promoting transcription factors (EMT-TFs). EMT-TFs from the Snail, Twist, and ZEB families are responsible for repressing epithelial genes and activating mesenchymal genes (4). The role of EMT in the metastatic cascade has been controversial. Although studies indicate that it is involved in basal membrane rupture, intravasation, resistance to shear stress in blood vessels, and extravasation, some studies have shown that EMT is not essential for metastatic colonization (5). A probable explanation is the multiplicity of possible outcomes for cells undergoing EMT. There are several intermediate stages in this process that contribute to the formation of subpopulations that differ in proliferation, invasion, plasticity, and metastatic capabilities. The plasticity allows cells to undergo reversible changes between epithelial and mesenchymal features, adapting to diverse hostile conditions (6). In this context, studies have attributed that cells in EMT display an increased resistance to different therapeutic treatments, with consequent disease relapse leading to patient death (7). The properties, mechanisms and proteins that regulate cellular events involved in the EMT program could become promising markers and/or therapeutic targets for cancer therapy to prevent metastasis. The implications of use as markers will be addressed in this chapter.

EMT-ASSOCIATED CELLULAR ALTERATIONS

EMT involves a range of alterations in cell morphology, gene expression, and physiology. However, evidence has shown the existence of cell programming stages

between the two poles of EMT (complete epithelial or complete mesenchymal), in which there is incomplete suppression of pre-existing epithelial characteristics and incomplete acquisition of mesenchymal features. This gave rise to the concept of a hybrid intermediate stage known as partial EMT, which has been observed within tumors in a broad range of cancers, including CRC (6). Upon activation of EMT, tumor cells undergo a series of physical changes, including tight junction dissolution, disruption of apicobasal polarity, and reorganization of the cytoskeletal architecture. These events facilitate the dissemination of cells from their primary site, invasion of surrounding tissues, survival in the general circulation, and ultimately lead to the formation of metastases. Morphologically, this switch leads to a striking loss of the typical polygonal, cobblestone appearance of epithelial cells and the emergence of spindle-shaped fibrous cells that express mesenchymal cell markers (8). Here, we present evidence related to two of these cellular EMT-related events: cell-cell adhesion disruption and cell polarity loss, as well as actin cytoskeleton reorganization (Figure 1).

Cell-cell adhesion disruption and cell polarity loss

Colonic epithelial cells are cohesive sheets of polarized cells maintained by specialized intercellular junctions constituted by the apical junctional complex (AJC). AJC is connected to the actin cytoskeleton and maintains the dynamic properties of this complex, tissue architecture, and cell homeostasis (9).

The establishment of apical-basal polarization is intimately linked to AJC, which is formed by adherens junctions (AJs) and tight junctions (TJs). TJs are composed of transmembrane proteins such as claudins, occludins, and junctional adhesion molecules (JAMs), whereas their cytoplasmic components are zonula occludens (ZOs) proteins. Basal to TJs, AJs bind cytoskeletal actin to the plasma membrane, creating an adhesive attachment between epithelial cells or the extracellular matrix and these cells. AJs consist of two basic adhesive units: the transmembrane component E-cadherins/nectins and the cytoplasmic component β-catenin/afadin complexes. Epithelial cells display an asymmetrical distribution of molecules, organelles, and structures, which is defined as cell polarity. Cell polarity is a fundamental process involved in many biological processes that contribute to normal tissue integrity and development. To allow correct sheet alignment, epithelial tissues are organized according to two polarity axes: apicobasal cell polarity (ABP) and planar cell polarity (PCP). The ABP orients cells from the free surface or the lumen to the basal lamina contributing to the acquisition of a cell shape that characterizes epithelial functions. The epithelial cell polarity is established and maintained through the concerted actions of three conserved polarity complexes: Par (Par3, Par6, aPKC and Cdc42), Crumbs (Crb, PATJ and Pals1), and Scribble (Scrib, Lgl and Dlg). ABP polarity is involved in diverse cellular pathways that control cell proliferation, apoptosis, and invasion (10, 11). Most proteins involved in the core ABP machinery exhibit alterations during epithelial transformation and are therefore implicated in human cancers (12).

Loss of cell polarity is one of the hallmarks of epithelial human cancers, and leads to tissue disorganization, increased proliferation, and metastasis (13). Indeed, loss of the polarity protein Pals1 in CRC cells enhances cell migration and invasion *in vitro* and increases the metastasis of transplanted tumor cells in mice (14). Additionally, loss of epithelial markers, including E-cadherin and

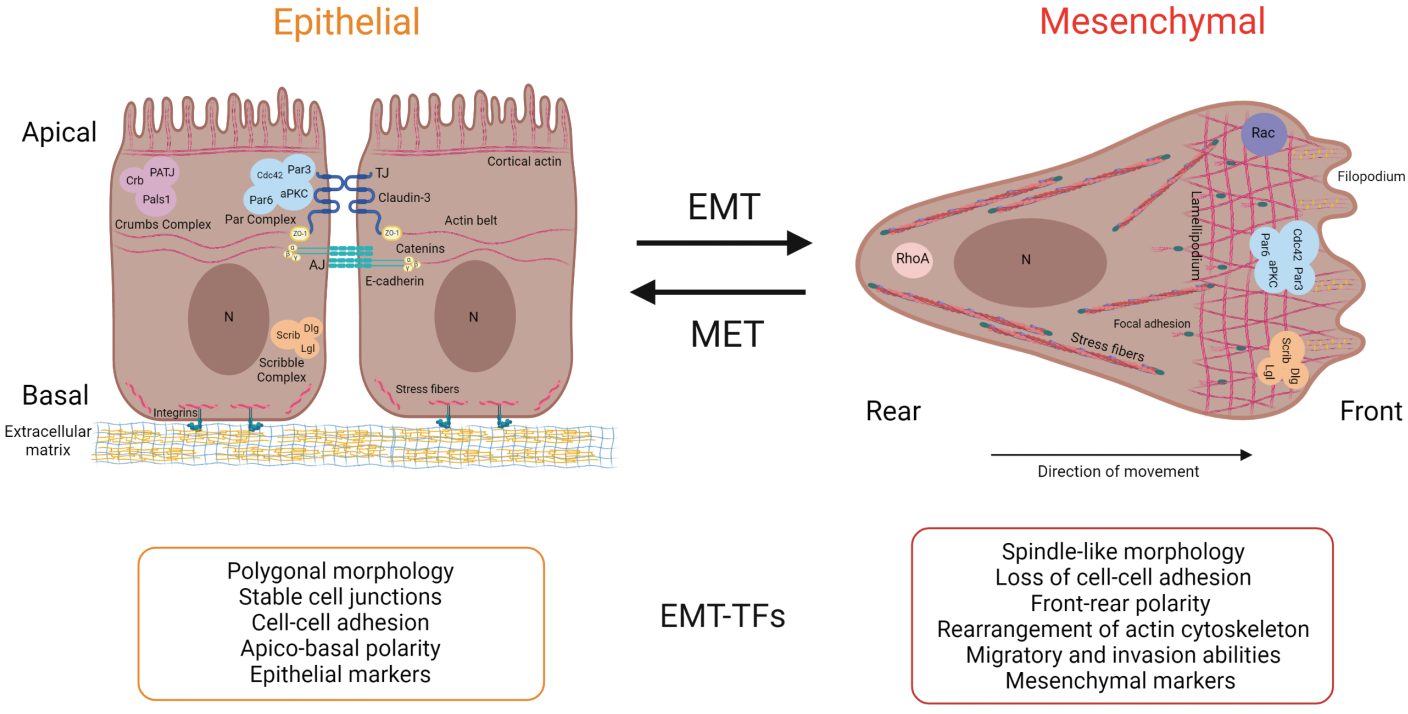

Figure 1. Schematic of overall EMT process. During EMT, epithelial cells change morphology to spindle-like shape accompanied by loss of cell polarity and cell-cell contacts, leading to rearrangement of actin cytoskeleton and acquisition of migratory and invasive abilities. Also, as a reversible process, MET could generate epithelial cells. AJ, adherens junctions; EMT, epithelial–mesenchymal transition; EMT-TFs, EMT transcriptional factors; MET, mesenchymal–epithelial transition; TJ, tight junctions. The image was produced using BioRender.com.

claudin–1, –3, and –7 proteins, is frequently observed during EMT (15). Low levels of claudin-1 in CRC promote EMT through NF-κB activation and correlate with aggressive tumor behavior (16). Moreover, downregulation of claudin-3 induces IL6/gp130/Stat3 signaling by hyperactivation of Wnt/β-catenin in CRC cells (17). In addition, loss of claudin-7 expression promotes EMT, invasion, and metastasis in CRC (18). It is almost certain that the downregulation of E-cadherin and the increased expression of vimentin are classic markers of EMT, which increases cell motility and facilitates invasiveness and metastasis. However, it has been showed that loss of E-cadherin is not an essential step for tumor invasion and different studies have reported that epithelial cells can collectively invade and colonize distant tissues and organs (19). Furthermore, E-cadherin is required for epithelial dissemination, and many metastases continue to express this protein (20). In fact, analysis of circulating tumor cells (CTCs) isolated from cancer patients showed simultaneous expression of epithelial and mesenchymal markers, indicating that cell dissemination appears to be a multi-stage process (21). Recently, it was shown that E-cadherin is internalized by annexin A2 protein, which contributes to the development of EMT in CRC cells (22). All the alterations described above, such as loss of cell polarity and cell-cell adhesion disruptions, are simultaneously connected to actin cytoskeleton rearrangement in the early steps of EMT.

Actin cytoskeleton reorganization

Cells undergoing EMT acquire increased migration and invasion potential, which is accompanied by actin cytoskeletal rearrangements. Actin organization is a dynamic process regulated by GTPases of the Rho family, such as Rho, Rac, and Cdc42, which are responsible for the formation of stress fibers, lamellipodia, and filopodia, respectively (23). The actin cytoskeleton also regulates many cellular events including cell migration, invasion, membrane trafficking, survival, proliferation, and polarity, which are crucial events in EMT. During growth factor-induced EMT, Rac1 activation regulates the formation, extension, and stabilization of lamellipodia at the front of cells. EMT-associated signaling pathways such as PAKs, NF-κB, MAPKs, Wnt/β-catenin/TCF, and STAT3 can also be triggered by Rac1 activation (24). The non-Smad TGF-β signaling pathway is involved in EMT, downregulating RhoA levels by ubiquitylation and modulating TJ and cell polarity assemblies. Additionally, at the leading edge of the cell, Cdc42 activates the Arp2/3 complex, contributing to the lamellipodium extension and directional sensing of migrating cells by regulating filopodial protrusion formation (25). Filopodia are finger-like actin-rich membrane protrusions composed of an actin binding protein called fascin-1 and cross-linked actin fibers. Fascin-1 is an actin turnover regulator and an important target in the EMT context, particularly associated with metastatic potential (26).

RhoA activates ROCK/LIMK/cofilin-1 signaling, which induces actin stress fiber formation, a contractile actin filament composed of α-actinin and myosin, impacting cell-cell adhesion disassembly and the turnover of focal adhesion, as well as the traction forces needed for cell motility (27). ROCK1 protein regulates the phosphorylation of myosin light chain (MLC), and its overexpression is associated with tumor progression and low five-year survival in CRC patients (28). LIMKs and SSH1 are proteins that regulate cofilin-1 by phosphorylation, modulate actin dynamics, and contribute to tumor aggressiveness

and metastasis of CRC cells. Recently, it was shown that during EMT, cofilin-1 promotes actin rearrangement, leading to cell-cell adhesion disruption and, consequently, cell migration and invasion are stimulated (15). Moreover, LIMK1 and SSH1 are upregulated in the mesenchymal consensus molecular subtype (CMS4) of CRC, and patients with high levels of LIMK1 exhibit low overall survival rates in canonical (CMS2) and metabolic (CMS3) subtypes (29). RhoA activation induces focal adhesion autophosphorylation and increases cell migration (30). In addition, the association of α-actinin 4 with increased motility and downregulation of junctional proteins supports its role in regulating the E-cadherin/β-catenin complex disassembly of CRC cells in EMT (31). In contrast, cortactin is frequently used as an EMT marker because it regulates protease activity in actin-rich protrusions that are essential for matrix degradation and invasion of tumor cells (32). The role and potential clinical value of these actin regulator proteins during cell migration and invasion during EMT are still limited.

EMT GENETIC ALTERATIONS

The EMT program is activated by autocrine and paracrine signals from the tumor microenvironment, which include a variety of cytokines, interleukins, and growth factors that stimulate signaling pathways in tumor cells, converging in the activation of a group of transcription factors. In addition to EMT-TF regulation of genes associated with epithelial and mesenchymal states, EMT is also regulated by non-transcriptional mechanisms through microRNAs, alternative splicing, epigenetic modifications, and post-translational modifications, which affect protein stability and localization (33). Two topics will be assessed in this section: transcription factors (EMT-TFs) and post-translational modification involved in EMT.

Transcription factors driving the EMT

Three major groups of transcription factors regulate the EMT program: the SNAIL family of zinc-finger, the zinc finger E-box binding homeobox (ZEB) family, and the TWIST family of basic helix-loop-helix (bHLH). The expression of these three groups promotes the regulation of early events in the EMT program through the repression of the epithelial phenotype and activation of the mesenchymal phenotype. Other transcription factors have been shown to influence EMT, such as, family of prospero homeobox 1 (PROX1) and forkhead box (FOX) transcription factors (34). These factors often regulate the expression of each other and functionally cooperate to regulate EMT. Their activities are localized in the nucleus, where they have access to the DNA to coordinate a cascade of signaling, leading to disruption of cell adhesions, loss of cell polarity, and manifestation of a mesenchymal motile phenotype (33). Moreover, EMT-TFs are linked to the induction of many other events, particularly stemness, survival, and changes in cell metabolism (35). The contribution of EMT-TFs in activating the EMT program depends on the cell or tissue type involved and the induction of signaling pathways present in the tumor microenvironment.

The role of these transcription factors in EMT has been well established in various cancers, including CRC. Aberrant activation of Wnt/β-catenin signaling is

common in patients, and nuclear accumulation of β-catenin results in the upregulation of EMT-TFs, mainly Snail, a core regulator of EMT that represses E-cadherin and promotes metastasis and invasion (36). The regulation of EMT-TFs is clinically relevant in metastasis because their expression is associated with an increased rate of cancer recurrence and decreased survival in CRC patients (37). Therefore, EMT-TFs are critical factors in regulating EMT, which lead to cancer metastasis. The Snail family consists of three transcription factors, Snail (also referred to as Snail1), Slug (Snail2), and the less characterized Smuc (Snail3), all of which repress epithelial genes by binding to E-box DNA sequences through their carboxy-terminal zinc-finger domain. Snail promotes EMT by repressing E-cadherin binding to the E-box sequence in the promoter region and recruits the polycomb repressive complex 2 (PRC2), which cooperates with reduced expression by epigenetic regulation (38). Furthermore, Snail increases the expression of genes related to the mesenchymal phenotype, such as fibronectin, vimentin, and N-cadherin, in addition to upregulating other EMT-TFs, including Slug, Zeb1, and Twist (39). Many signaling pathways, such as, TGF-β, PI3K/Akt and NOTCH, initiate the progression of EMT via the expression of higher levels of SNAIL (8). In CRC, Wnt/β-catenin signaling and Snail cooperate to induce EMT by upregulating LEF1, leading to increased interaction with β-catenin to promote invasion (40).

Twist1 and Twist2 belong to a group of factors that share a basic helix (loop) protein structure. Twist1 represses E-cadherin and induces N-cadherin expression, probably through its association with methyltransferase SET8 (also known as SETD8), which mediates monomethylation of histone marks (41). The ZEB family consists of two members, Zeb1 and Zeb2, which bind to regulatory gene sequences in E-boxes. Structurally, Zeb proteins contain two zinc finger clusters localized at the N- and C-terminal that bind to E-boxes (42). Zeb1 and Zeb2 are two of the leading EMT regulators that directly bind to the E-box sequence in the E-cadherin promoter region or indirectly form a repressor complex with epigenetic proteins. Therefore, Zeb1 recruits histone deacetylase or methyltransferase to decrease E-cadherin expression and activate the EMT program (43). In addition, Zeb1 triggers the loss of basal-apical polarity by directly suppressing the activation of polarity factor genes as a consequence of increased metastasis (44). These interrelationships lead to numerous permutations that need to be resolved for a better understanding of EMT in colorectal cancer.

Post-translational modifications

Post-translational modifications (PTMs) drastically increase the diversity of protein structures and play a fundamental regulatory role in cell physiology. PTMs occur in amino acid side chains or peptide bonds and are usually mediated by enzymes. It is estimated that approximately 5% of the genome encodes enzymes that are responsible for PTMs (45). There are more than 400 types of PTMs, and the most common include phosphorylation, ubiquitylation, glycosylation, and acetylation (46). Transcription factors (TFs) that regulate EMT are regulated by phosphorylation (47). For instance, the main mechanism involved in the control of Snail stability is phosphorylation at Ser residues promoted by glycogen synthase kinase 3 beta (GSK-3β), which induces its nuclear export and generates a binding site for E3 ubiquitin ligase, known as beta-transducin-repeat-containing protein

(β-TrCP). In contrast, SCP family phosphatases remove these phosphorylations, promoting Snail stabilization (48). Another protein that can phosphorylate Snail is protein kinase D1 (PKD1); in this case, after phosphorylation at Ser11, Snail is recognized by SCF-FBXO11 ubiquitin ligase, thus being targeted for proteasomal degradation (49). These data indicate that phosphorylation and ubiquitylation act in an interconnected manner to regulate EMT.

Glycosylation is the enzymatic addition of sugars to proteins and lipids. The two types of glycosylation that are most often found in proteins are *N*-glycosylation and *O*-glycosylation. *N*-linked glycans are attached to nitrogen atoms present in the side chain of asparagine (Asn) residues that constitute the Asn-X-Ser/Thr consensus sequence, where X can be any amino acid, except proline. In turn, *O*-linked glycans are attached to the oxygen atoms of the Ser or Thr residues. During EMT, the levels of several glycans are altered, which permit regulation of the mechanisms involved in this event. In TGF-β-induced EMT in mouse mammary gland cells, decreased levels of bisecting *N*-acetylglucosamine (GlcNAc) structures, a modification characterized by the attachment of GlcNAc to the core mannose of *N*-glycans in a β1,4-linkage catalyzed by the MGAT3 (or GnT-III) enzyme, was observed (50). The overexpression of *MGAT3* resulted in the inhibition of hypoxia-induced EMT in MCF7 breast cancer cells. While bisecting GlcNAc structures have been associated with reduced malignancy and EMT suppression, the β1,6-GlcNAc branched *N*-glycans (structure formed by the transfer of a GlcNAc residue to the α1,6-mannose via the β1,6-linkage), which are catalyzed by MGAT5 (or GnT-V), have been associated with malignancy and EMT promotion (Figure 2) (51).

Key proteins that control EMT are also regulated by acetylation, with lysine (Lys) residues being frequently observed. In breast cancer cells, CREB-binding protein (CBP or CREBBP), a histone acetyltransferase (HAT), acetylates Slug at Lys211 and Lys166, thus contributing to the stabilization of this TF and consequently promoting EMT (52). CBP acetylates Snail at Lys146 and Lys187, which prevents Snail from interacting with a repressor complex that includes HDAC1, HDAC2, and Sin3A proteins, leading to an increase in pro-EMT gene expression (53).

These results of the studies detailed above show that the functionality of EMT master regulators is tightly regulated by PTMs. However, further research in this area is needed to understand this complex cellular process.

EMT AND THERAPY RESISTANCE

Complete loss of epithelial features concomitant with the gain of a fully mesenchymal phenotype during EMT is rare in human carcinomas. Normally, cells with high plasticity exhibiting both epithelial and mesenchymal features between different intermediate phenotypic states of EMT are observed. This plasticity promotes increased tumor heterogeneity and interaction with tumor-associated stromal cells, which exhibit pro-tumorigenic properties, and seem to be responsible for the mechanism of resistance to therapeutic treatments (e.g., radioresistance and chemoresistance); and the development and maintenance of a tumor stem phenotype (54).

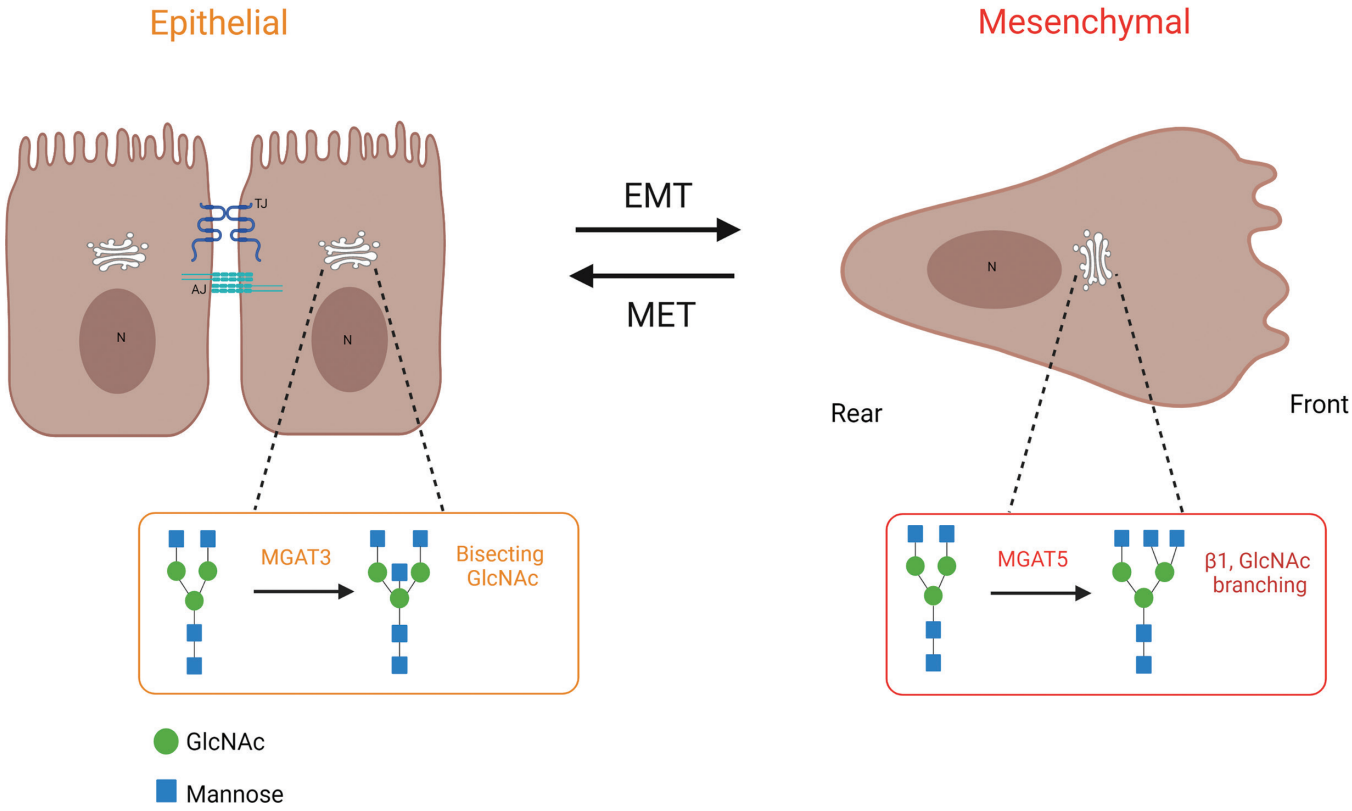

Figure 2. Glycosylation reactions catalyzed by the enzymes MGAT3 (GnT-III) and MGAT5 (GnT-V). Increased levels of bisecting GlcNAc *N*-glycans have been related to the epithelial phenotype, while in cells that underwent EMT, high levels of β1,6-GlcNAc branched *N*-glycans are observed. EMT, epithelial-mesenchymal transition; MET, mesenchymal–epithelial transition; MGAT3, *N*-acetylglucosaminyltransferase III; MGAT5, *N*-acetylglucosaminyltransferase V. The image was produced using BioRender.com.

Radioresistance

Radiotherapy (RT) is used for treatment with curative or palliative intent; however, resistance to this treatment is the main reason for therapeutic failure, which can lead to tumor recurrence and metastasis. In this context, some studies have shown that RT can induce EMT, thus leading to the acquisition of a resistant phenotype with decreased activation of cell death pathways by apoptosis (55). In CRC cells, it was shown that the progeny derived from radioresistant cells developed an EMT-like phenotype characterized by reduced E-cadherin expression, overexpression of β-catenin and vimentin, and increased cell migration and invasion (56). In a subsequent study using the same cellular progeny, EPHA4 receptor activation was found to be upstream of the PI3K/AKT, Wnt/β-catenin, and ERK1/2 pathways, playing an important role in the regulation of events related to EMT (57). Another study using CRC cells showed RT induced EMT by increasing ROS and activating AKT/Src/ERK signaling, resulting in increased Snail and decreased E-cadherin expression (58). These results indicate that these events may be associated with therapeutic failure in CRC after RT.

Reactive Oxygen Species (ROS), cellular components of the tumor microenvironment, and a hypoxic condition, mainly through hypoxia-inducible factor (HIFs), play an important role in the activation of signaling pathways that induce EMT and tumor resistance. Some of these pathways include TGF-β, Wnt/β-catenin, NOTCH, EGFR, NF-κB, IL-6/STAT3, PI3K-AKT, and ERK. Likewise, specific miRNAs, lncRNAs, and circRNAs also contribute to the induction and maintenance of EMT-associated resistant phenotypes (59). In addition, some of these converge with the cancer stem cells (CSCs) activation pathways, where the induction of EMT in non-stem cells can promote radioresistance through the gain of stem-like characteristics. The mechanism linking these two events has not been fully elucidated. However, it is possible that proteins secreted by cells during EMT act in an autocrine manner, leading to the induction and maintenance of the CSC phenotype. The resistance associated with these cells has been attributed to mechanisms such as the decreased expression of pro-apoptotic proteins, increased expression of anti-apoptotic proteins, low proliferation rate, and evasion of the immune system (60). Some studies have shown that EMT negatively regulates senescence and vice versa. Thus, cyclin-dependent kinase inhibitors (CDKIs) inhibit EMT-TFs, whereas those in EMT show decreased expression of classic senescence markers, such as p21/p27 and p14/p16, through repression of the transcription of these proteins mediated by EMT-TFs (61). In fact, EMT induction or senescence in CRC cells after RT treatment did not describe the simultaneous occurrence of both phenotypes (62).

EMT is also related to other cellular events that contribute to radioresistance, such as the formation of polyploid giant cancer cells (PGCCs). These cell types display both EMT and CSC characteristics and can generate daughter cells with these properties (63). Autophagy is another known RT resistance mechanism. Autophagy and EMT share signaling pathways such as RAS/RAF/MEK/ERK, beclin-1, and TGF-β, in which their mediators positively activate both mechanisms (64). It is possible that the events mentioned above may be responsible for therapeutic failure after RT in patients with CRC; however, the post-irradiation behavior and phenotype of radioresistant cancer cells remain largely unknown. Thus, the identification of biomarkers and altered pathways in RT-resistant cells may help improve clinical efficacy.

Chemoresistance

Many tumors present innate resistance before drug treatment (intrinsic resistance) or can become resistant after long-term treatment with these drugs (acquired resistance). This phenomenon of resistance by tumor cells to different anticancer drugs is termed multidrug resistance (MDR) and is a major challenge in modern cancer treatment. MDR results in simultaneous cross-resistance to multiple unrelated chemotherapeutic agents, and is associated with treatment failure, poor survival, and disease recurrence. One of the most important mechanisms underlying MDR is the overexpression of adenosine triphosphate (ATP)-binding cassette (ABC) transporters that efflux anticancer drugs against a concentration gradient using ATP-driven energy. These pumps alter enzymes responsible for drug metabolism, enhance DNA repair response, alter the microenvironment and survival of CSCs, and activate EMT (65).

A link between EMT and chemoresistance was proposed in the early 1990s (66). Subsequently, numerous cancers have been reported to overexpress EMT markers after treatment with various chemotherapeutic agents (67). Using an EMT lineage-tracing system in transgenic mice with breast cancer, cyclophosphamide resistance was associated with Zeb1 and Zeb2 expression, reduced proliferation, and apoptotic tolerance (68). Snail or Twist suppression in mouse models of pancreatic ductal adenocarcinoma resulted in higher levels of nucleoside transporters in tumors, contributing to increased sensitivity to gemcitabine (69). In lung cancer cell lines, cisplatin resistance is mediated by Slug, which inhibits apoptosis by suppressing the proapoptotic protein PUMA (70). In addition, Snail overexpression has been associated with evasion of apoptosis, MDR phenotype, enhanced P-glycoprotein levels, and increased cell stemness in breast cancer cell lines (71). Patients displaying the mesenchymal subtype of CRC (CMS4) exhibited chemoresistance profiles in response to Hsp90 inhibitors in preclinical models (72) and unresponsiveness to adjuvant 5-fluorouracil (5FU) therapy in a clinical cohort (73).

The molecular mechanisms involved in chemoresistance are complex, as multiple signaling pathways can contribute in different ways and are tissue specifics. TGF-β, Hedgehog, Notch, Wnt/β-catenin signaling, and FOX transcription factor superfamily members are related to chemoresistance associated with EMT in diverse cancers. For instance, blocking TGF-β signaling by inhibiting Smad4 abolished doxorubicin resistance in a CRC cell line (74). Increased nuclear β-catenin was detected in the mesenchymal tissues of CRC patients resistant to doxorubicin, and was associated with low levels of E-cadherin, high levels of Snail, and expression of CSC-related markers (75). Indeed, Wnt/β-catenin signaling has been shown to regulate the transcription of *MDR1* (P-glycoprotein) via TCF/LEF, pro-survival signaling, expression of CSCs marker genes, and normal stem cell differentiation and proliferation in intestinal crypts (76). CSCs properties are also associated with chemoresistance and EMT, sharing gene expression signatures and key signaling pathways. CSCs possess mechanisms of adaptation that mediate their growth, survival, and chemoresistance. These mechanisms include slow proliferation and increased levels of efflux pumps that permit the elimination of cytotoxic drugs and, consequently, exhibit a high ability to perpetuate the tumor after this therapy (77).

Different microenvironmental tumor factors, such as immune cells, cancer-associated fibroblasts (CAFs), hypoxia, matrix extracellular compounds, interleukins, and growth factors also play key roles in driving EMT, chemoresistance, and CSCs, and these components interact to form a permissive niche for tumor progression. A study using CRC patient-derived models showed that under hypoxia, CAFs secrete TGF-β2 and induce the expression of hedgehog transcription factor (GLI2) via HIF-1α in CSCs, promoting 5FU and oxaliplatin chemoresistance (78). Overall, these studies demonstrated that CRC chemotherapy is also intimately linked to the EMT molecular signature and an enriched CSCs population, contributing not only to relapse but also to resistance and metastasis.

EMT COMPONENTS AS TARGETS TO INHIBIT METASTASIS

Owing to their crucial role in processes such as tumor progression, migration, invasion, and therapy resistance, EMT components have become a target for researchers looking for ways to stop or at least hinder the metastatic cascade. However, there are three main complications associated with targeting EMT to inhibit metastasis. First, while targeting EMT-TFs or mesenchymal-specific proteins, another important cell population in the tumor, the fibroblasts, might be affected. These cells are active regulators of paracrine signaling and structural remodeling of the tumor microenvironment but are also essential for the maintenance of normal epithelial function across the digestive tract. Second, EMT plasticity with all its intermediate stages that are employed at each step of the metastatic cascade poses the persistent question of finding the correct stage to target (79). Finding a drug that inhibits the expression of mesenchymal characteristics might stop tumor cells from further invasion but may also promote metastatic colonization from early metastasized cells. Third, there is considerable redundancy in EMT-related pathways, with multiple EMT-TFs regulating specific genes and several pathways leading to this program (33). Therefore, a single-target strategy is most likely to be inefficient in a clinical setting. However, with multi-target therapies, unintended side effects from the interconnected pathways that participate in the EMT program are also crucial to other physiological processes in healthy cells.

A few targets such as natural compounds, small-molecule inhibitors, and monoclonal antibodies, have already been assessed in preclinical studies. TGF-β is an important EMT inducer and one of the main targets for EMT prevention. However, owing to its multiple receptors and canonical and non-canonical downstream signaling, inhibiting TGF-beta-induced EMT poses a serious challenge. Natural compounds such as resveratrol (80) and small-molecule inhibitors have been successful in preclinical studies, but none have reached human trials for CRC. Monoclonal targeting of TGF-beta receptors, fresolimumab, has been attempted for renal cell carcinoma and melanoma in a phase I trial, with no significant improvement in patient survival (81). It is important to highlight that TGF-β plays a dual role acting as tumor promoter or suppressor in CRC progression, depending on the activation status of its signaling pathways. As a tumor suppressor it acts in the early stages of tumorigenesis. However, it can also promote invasiveness and metastasis in advanced stages of tumor progression (82). Therefore, specific targeting might have unfavorable outcomes depending on the tumor stage. Multityrosine kinase inhibitors, such as cabozantinib and

regorafenib, have shown some success in clinical trials, with the latter being approved by the Food and Drug Administration (FDA) of the United States as a last resort for the treatment of metastatic CRC. Regorafenib abolishes EMT-induced invasion and metastasis through the activation of SHP-1 tyrosine phosphatase (83). Cabozantinib interferes with AXL signaling, downregulates EMT-TFs, and increases E-cadherin expression. However, in a combined treatment with the EGFR inhibitor panitumumab for KRAS wild-type CRC patients, cabozantinib showed a 16% response in terms of clinical parameters (84). Methotrexate successfully increased E-cadherin expression levels 10-fold in CRC cell lines, and it has been shown to be ineffective in clinical settings (85). The antibiotic salinomycin has also been shown to downregulate vimentin and prevent EMT induction by doxorubicin (86). However, targeting EMT effectors such as E-cadherin and vimentin, as previously discussed, may result in an increased number or metastatic size if we consider the reverse process of EMT, which is a crucial step in secondary tumor formation.

Considering all these characteristics, perhaps the most promising strategy for EMT targeting is an indirect approach. Repurposing already approved metabolic drugs has shown a significant EMT-inhibiting potential (87). The high potential of utilizing the increased cell platicity inherent to invasive cancer cell instead of avoiding EMT represent an innovative technique of transdifferentiation therapy. For instance, Ishay-Ronen et al. have shown that, for breast cancer cells, cell proliferation and EMT plasticity can stop inducing terminal differentiation in cancer-derived adipocytes through a combined treatment with rosiglitazone (PPARγ agonist) and trametinib (MEK inhibitor). This promising transdifferentiation strategy reduced invasion and metastasis of breast cancer in animal models (88). However, the available experimental evidence for colorectal cancer is still scarce compared with other tumor types.

CONCLUSION

EMT is a highly orchestrated program through a complex network of numerous regulatory factors and cell signaling pathways that interact with each other to regulate crucial processes involved in tumor progression. In recent years, a series of studies have facilitated the establishment of various EMT markers that are being used in preclinical models to inhibit the effect of EMT on invasiveness, metastasis, and drug resistance. However, the complex heterogeneity of tumors and the reversible plasticity of EMT, which is fundamental for tumor progression and drug resistance, are principal obstacles in the reproduction of clinical cancer progression. Therefore, further research to establish new biomarkers for EMT, with special emphasis on partial EMT as well as a better understanding of how EMT and therapy resistance are interconnected, is imperative for the development of new therapeutic approaches for CRC.

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