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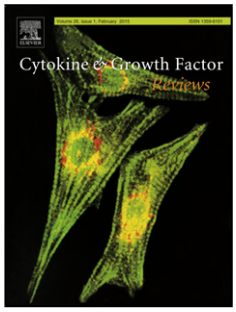

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Deciphering the loop of epithelial-mesenchymal transition, inflammatory cytokines and cancer immunoediting.

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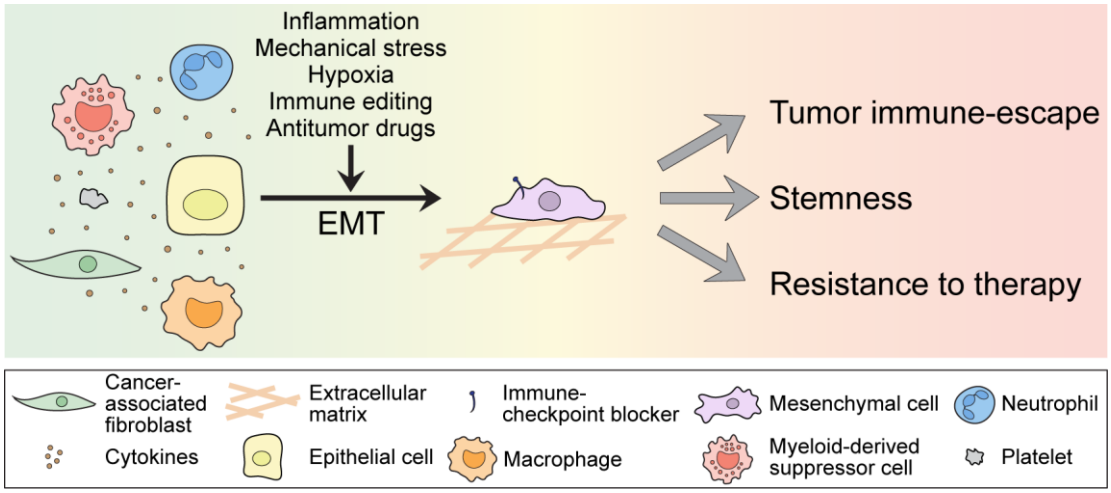
Running title: Cross-talks during epithelial-mesenchymal transition: the allies to disarm antitumor immune defences.

Graphical abstract



Highlights

- Tumorigenesis and tumor progression depend on cell-cell interactions, cell-extracellular matrix interactions and soluble cues (*i.e.*, cytokines and growth factors).
- The immune system shapes tumor fate by activating innate and adaptive mechanisms.
- The phenotypic plasticity is a common feature of epithelial and immune cells.
- The epithelial–mesenchymal transition (EMT) is a physiological and coordinated program in which epithelial cells acquire motile phenotype and the characteristics of mesenchymal cells.
- In tumors, EMT is a leading biological process regulating cancer invasion, metastasis, immune escape and resistance to therapy.
- In tumor cells EMT is induced by the cooperation and convergence of multiple signaling pathways activated by the dialogue between tumor cells and stromal cells into the tumor microenvironment, related to an inflammatory response.
- The activation of EMT program increases cell phenotypic heterogeneity and stemness, with important implications for the resistance to chemotherapy, targeted therapies and immunotherapy.

Abstract

Tumorigenesis and tumor progression relies on the dialectics between tumor cells, the extracellular matrix and its remodelling enzymes, neighbouring cells and soluble cues. The host immune response is crucial in eliminating or promoting tumor growth and the reciprocal coevolution of tumor and immune cells, during disease progression and in response to therapy, shapes tumor fate by activating innate and adaptive mechanisms. The phenotypic plasticity is a common feature of epithelial and immune cells and epithelial-mesenchymal transition (EMT) is a dynamic process, governed by microenvironmental *stimuli,* critical in tumor cell shaping, increased tumor cell heterogeneity and stemness. In this review we will outline how the dysregulation of microenvironmental signaling is crucial in determining tumor plasticity and EMT, arguing how therapy resistance hinges on these dynamics.

Abbreviations:

AKT1/AKT: AKT serine/threonine kinase 1;

AXL: AXL receptor tyrosine kinase;

BMI1: BMI1 proto-oncogene, polycomb ring finger;

CAF: cancer-associated fibroblast;

CDH/CAD: cadherin;

CRKL: CRK Like Proto-Oncogene;

CSC: cancer stem cell;

CSF-1: colony-stimulating factor-1;

CTLA4: cytotoxic T-lymphocyte antigen 4;

CXCL: C-X-C motif ligand;

CXCR: C-X-C chemokine receptor;

ECM: extracellular matrix;

EGF: epidermal growth factor;

EMT: epithelial–mesenchymal transition;

ESRP: epithelial splicing regulatory protein;

FGF: fibroblast growth factor;

FSP-1: fibroblast-specific protein-1;

HDAC3: histone deacetylase 3;

HGF: hepatocyte growth factor;

HIF-1α: hypoxia-inducible factor-1α;

ICB: immune checkpoint blocker;

IFN-γ: interferon-γ;

IL: interleukin;

IRAK-M: interleukin-1 receptor-associated kinase-M;

JAK: Janus kinase;

KRAS: Kirsten rat sarcoma viral oncogene homolog;

LOXL2: lysyl oxidase 2;

MDSC: myeloid-derived suppressor cell;

MET: mesenchymal-epithelial transition;

MHC-I: major histocompatibility complex-I;

miRNA: microRNA;

MMP: metalloproteinase;

NF-κB: nuclear factor-κB;

NLRP3: NACHT, LRR and PYD domains-containing protein 3;

NSCLC: non-small cell lung cancer;

PDGF: platelet-derived growth factor;

PD-L1: programmed death-ligand 1;

RAC1: ras-related C3 botulinum toxin substrate 1;

ROS: reactive oxygen species;

α-SMA: α-smooth muscle actin;

SMAD2: SMAD family member 2;

SNAI1/SNAIL: snail family transcriptional repressor 1;

SNAI2/SLUG: snail family transcriptional repressor 2;

SOX9: SRY-box 9;

STAT3: signal transducer and activator of transcription 3;

TAM: tumor-associated macrophage;

TAZ: tafazzin;

TGF-β: tumor growth factor-β;

TME: tumor microenvironment;

TNF-α: tumor necrosis factor-α;

TP53/p53: tumor suppressor p53;

TWIST1/TWIST: twist family bHLH transcription factor 1;

VEGF: vascular endothelial growth factor;

YAP1/YAP: yes associated protein 1;

ZEB1: zinc finger E-box-binding homeobox 1.

Keywords: EMT; tumor microenvironment; tumor immunoediting; CSC

Introduction. Epithelial-mesenchymal transition at a glance.

The epithelial–mesenchymal transition (EMT) is a highly conserved, naturally occurring transdifferentiation program that governs changes in cell states along the epithelial *versus* mesenchymal axis, conferring epithelial–mesenchymal plasticity. Known from embryologists as soon as 1879 [1], EMT has been of interest to the research community since Greenburg and Hay first described a mesenchymal-like transformation of epithelial cells when suspended in collagen gels [2]. From then on, EMT and the reverse process, termed mesenchymal-epithelial transition (MET) are recognized as the foremost trans-differentiation programs operating during development and ensuring a proper histogenesis and organogenesis, through plastic interconversions between epithelium and mesenchyme [3]. In adults, EMT assists tissue regeneration and regrowth during wound repair and guarantees the re-establishment of the epithelial integrity, essential for tissue homeostasis [4, 5]. When repair mechanisms are not properly executed, myofibroblasts induce fibrotic extracellular matrix (ECM), altering normal cell functions and tissue homeostasis. This leads to organ fibrosis, a tissue context facilitating tumor growth and progression [6]. Noteworthy,

EMT is widely recognized as a leading biological process regulating cancer invasion, metastasis and immune escape (**Figure 1**).

Despite being biologically equivalent, the physiological and pathological EMT follow different mechanistic rules - the first complying with non-inflamed highly regulated spatial and temporal plans, the latter instead mainly being an inflamed stochastic and time-independent process [7].

Conventionally, epithelial cells are defined as surface barrier cells with secretory functions that show distinct apical *versus* basolateral polarity established by desmosomes, adherent, tight and gap junctions with cell-ECM integration controlling the tissue architecture [8]. Conversely, mesenchymal cells are loosely organized, able to remodel ECM and to modify the integrin-ECM axis with consequent increased expression of metalloproteinases (MMPs) and acquired migratory and invasive ability [8] (**Table 1**).

The transition from the epithelial to mesenchymal cell state encompasses a spectrum of inter- and intra-cellular changes most likely determined by the integration of extracellular signals perceived by the cells (**Figure 1**). Indeed, the EMT program is mediated by complex signaling networks induced by different dynamic *stimuli* triggered by stromal cells and ECM components of the surrounding microenvironment and by soluble factors [epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), tumor growth factor (TGF)-β and vascular endothelial growth factor (VEGF)]. Other EMT inducers are the morphogens Wnt, Notch and Sonic hedgehog, and pro-inflammatory cytokines [*i.e.*, interleukin (IL)-6, IL-8, tumor necrosis factor (TNF)-α] [3, 9-11]. A paramount consequence of these pro-inflammatory and hypoxic responses is the upregulation of a number of transcriptional factors, repressors of epithelial genes or activators of mesenchymal genes, such as the snail family transcriptional repressor 2 (SNAI2, also known as SLUG), snail family transcriptional repressor 1 (SNAI1, also known as SNAIL), zinc finger E-box binding homeobox 1 (ZEB1) family members and twist family bHLH transcription factor 1 (TWIST1, also known as TWIST), all of which

repress cadherin 1 (*CDH1*, also known as E-cadherin) gene, a central caretaker of the epithelial phenotype. Concomitantly, mesenchymal markers, such as cadherin 2 (CDH2, also known as N-cadherin), vimentin, fibronectin, fibroblast-specific protein (FSP)-1 and α-smooth muscle actin (SMA) are upregulated [3, 12], and dramatic changes to the actin cytoskeleton [13], cell architecture and behaviour (**Figure 2**).

Besides the *stimuli* mentioned above and involving cytokine production and immune responses; mechanical stress; altered composition of the ECM; hypoxia and low pH and conditioning of antitumor drugs also concur to the EMT process [14] (**Figure 3** and **Table 2**).

Furthermore, epigenetics and alternative splicing program as well as microRNAs (miRNAs) all participate in EMT and MET [8] (**Figure 3** and **Table 2**). The alternative splicing, a process that allows the generation of multiple protein isoforms from a single gene, is frequently altered in cancer [15] and the identification of the epithelial splicing regulatory protein (ESRP)1 and ESRP2 revealed that alternative splicing provides an additional layer of regulation that mediates the transition between epithelial and mesenchymal cell states [16]. ESRPs are essential for maintaining the epithelial phenotype, and literature data have shown that TGF-β activates ZEB1 to repress ESRP expression and induce EMT [17]. A large proportion of the genes regulated by ESRPs, as well as by other EMT-related splicing factors, are involved in the actin cytoskeleton organization, cell–cell junctions, regulation of cell migration, and wound healing, all important for the EMT program [18]. A paradigmatic example of this regulation is the splicing program of the actin cytoskeleton regulator hMENA, with the switch from hMENA^{11a} to hMENAΔv6 isoform expression, as a crucial node in EMT-related signaling pathways [19-21] (**Figure 2**, **Table 1** and **Table 2**). In accordance, TGF-β1 down-regulates hMENA^{11a} and up-regulates hMENAΔv6 isoforms, a process crucial for SMAD family member 2 (SMAD2)-mediated TGF-β1 signaling and TGF-β1-induced EMT [21]. hMENA^{11a} is spliced by ESRP1/2 and its silencing reduces E-cadherin, whereas the overexpression of hMENAΔv6, increases vimentin expression. This is confirmed in primary breast tumors

hMENA^{11a} negative, that are more frequently E-cadherin low in comparison with tumors expressing hMENA^{11a} [19]. Of relevance in early non-small-cell lung cancer (NSCLC) the pattern of hMENA isoforms is a powerful prognostic factor with the expression of hMENA^{11a}, crucial in predicting disease recurrence and patient survival after surgery [20]. Similarly, in pancreatic cancer the hMENA^{11a} negative staining has been associated with poor overall survival [21].

The contribution of miRNAs, a class of noncoding small RNAs that can regulate the translational efficiency or stability of targeted mRNAs, in the regulation of EMT-transcriptional factors has been reported [22]. The TGF-β/ZEB/miR-200 autocrine signaling network has been shown to affect dynamic and reversible DNA methylation of the miR-200 family loci, which may influence epithelial-mesenchymal cell plasticity [23, 24]. Similarly, the miR-34 family induced by tumor suppressor p53 (TP53, best known as p53), suppresses the EMT-inducing transcription factor SNAIL expression and conversely, SNAIL and ZEB1 bind to the miR-34 promoters, thereby repressing its expression [25].

In this review, keeping in mind that phenotypic plasticity is a hallmark of tumor and immune cells, we will discuss the dynamic interplay between tumor cells and their microenvironment, with a particular emphasis on inflammatory cytokines, regulating an EMT program that propels tumor cell stemness and the acquisition of immune escape capabilities. Considering that the EMT process generates distinct tumor cell sub-populations, increasing intratumoral heterogeneity and resistance to therapies, we foresee that decodification of microenvironmental signaling will streamline the design of innovative, combined (immuno)therapeutic strategies.

Microenvironmental regulation of EMT: interdependency among tumor, stromal and immune cells, biochemical and biophysical players.

An inflammatory microenvironment.

Inflammation can affect every aspect of tumor development and progression as well as the response to therapy and is a critical component of the tumor microenvironment (TME) [26]. Established tumors, by engaging a dynamic dialogue with stromal and immune cells under the influence of inflammation-associated signaling exhibit EMT/MET plasticity to adapt to the changing surrounding environment [27]. This microenvironment, composed of inflammatory cells and inflammatory mediators, such as cytokines and chemokines, strongly contributes to the EMT program and disease progression [27] (**Figure 1**).

Among the cellular components, tumor-associated macrophages (TAMs) have a crucial role as major players in orchestrating cancer-related inflammation. TAMs, either the pro-inflammatory M1 or the pro-tumorigenic M2 subtypes, have been shown to induce EMT at the invasive front of tumors mainly through TGF-β production [28, 29] and stabilization of TNF-α-mediated SNAIL [30]. Noteworthy, cancer cells secrete the macrophage colony-stimulating factor (CSF)-1 to recruit TAMs which in turn, by releasing EGF, edit cancer cells to a mesenchymal phenotype favouring extravasation and metastasis at distant sites [31]. In agreement with this bidirectional communication, murine lung cancer cells produce TGF-β that induces the expression of IL-1 receptor-associated kinase (IRAK)-M in TAMs, promoting M2 polarization and thus tumor progression [32]. Myeloid-derived suppressor cells (MDSCs) and neutrophils have also been involved in tumor progression of different cancer types by producing TGF-β, EGF, and HGF, and by activating the C-X-C motif ligand (CXCL)-8 - C-X-C chemokine receptor (CXCR)-2 axis, respectively [33, 34].

In addition, platelets, whose primary role is to stop bleeding after tissue injury, have been shown to provide a TGF-β signaling platform in the bloodstream. This signaling, generated outside the primary microenvironment, confers a more mesenchymal phenotype and increased metastatic capacity to colon and breast circulating cancer cells [35].

The contribution of specific cytokines as inflammatory soluble mediators of the EMT program has been widely reported. TGF-β emerges as a master regulator of the pro-invasive TME and a potent

inducer of EMT [36]. Other than maintaining tissue homeostasis and suppressing inflammation and tumorigenesis, TGF-β can also activate and sustain inflammation favouring tumor progression depending on the cellular context [36, 37]. The complexity of the pathways activated by TGF-β signaling impedes a detailed description and is beyond the scope of this review; for a more extensive review refer to [37-39]. In general, in a tissue specific manner, TGF-β interplays with multiple cytokines and sustains pro-inflammatory circuits during the onset and progression of the EMT program [40]. Among the cytokines, IL-6 and TNF-α orchestrate a tumor-promoting microenvironment through effects on many different cell types. IL-6, a keystone cytokine in inflammation and cancer [41], is able to induce EMT *via* the Janus kinase (JAK)-signal transducer and activator of transcription 3 (STAT3)-induced SNAIL expression [10]. In a panel of estrogen receptor-α-positive human breast cancer cells IL-6 impairs E-cadherin expression and induces vimentin, N-cadherin, SNAIL and TWIST [42]. In primary colorectal tumors associated with a mesenchymal phenotype an IL-6R/STAT3/miR-34a feedback loop has been reported [43]. Of clinical relevance, IL-6 expression in serum or tissue samples of cancer patients correlated with poor prognosis [44, 45]. Given its established role in chronic inflammation, tissue remodelling, tumor growth, angiogenesis and metastasis, TNF-α is likely to be an important EMT-promoting cytokine in a variety of cancers. By inducing protein stabilisation of SNAIL and *β*-catenin, through nuclear factor (NF)-*κ*B and AKT serine/threonine kinase 1 (AKT1, also known as AKT) signaling pathways, TNF-α contributes to EMT induction and invasion in tumor cells [11]. In normal MCF-10 mammary epithelial cells, chronic treatment with TNF-α and IL-1β induces the destruction of normal breast acini architecture, EMT and spreading of non-transformed cells [46]. More recently, TNF-α and interferon (IFN)-γ have been shown to enhance the invasiveness of papillary thyroid cancer cells and this coincided with E-cadherin repression and N-cadherin and vimentin upregulation [47]. Similarly, IL-8, a pro-inflammatory chemokine upregulated in a wide variety of solid tumors, is involved in stemness, invasion and metastasis [48]. The role of IL-8 in invasion and metastasis raises from *in vivo* studies reporting that significant upregulation of IL-8 and increased

invasion occur in highly compared to poorly metastatic cells [49]. The IL-8-IL-8R axis has been shown to be essential for the maintenance of the mesenchymal, invasive phenotype of breast, lung and pancreatic tumor cells undergoing brachyury-mediated EMT [50].

Among the soluble immune players present in TME, the complement, a component of innate immunity, which also participates in the adaptive immune response, has been reported to be present in tumor tissue from patients with different cancers although its role in tumor progression is still unclear [51-53]. Recently, a close association between TWIST and the complement protein C3 has been shown. TWIST induces C3 expression in diverse murine tumor models and once produced, C3 acts as a negative regulator of E-cadherin expression, thus fostering EMT downstream of TWIST signaling [54]. Furthermore, C1q has been reported as contributing to tumor growth and invasion by acting as an external component of the extracellular matrix, independently of complement activation [55].

Cancer-associated fibroblasts (CAFs).

Among non-neoplastic cells recruited into the TME, CAFs are found in almost all solid tumors and may be derived from multiple sources during tumorigenesis. Although, secreted signal molecules distinguish differentiated CAFs from non-neoplastic fibroblasts, the molecular profile of these heterogeneous cells has not yet been defined. CAFs orchestrate key pathophysiological processes in cancer development through paracrine interactions involving the secretion of multiple soluble factors, the synthesis and remodelling of the ECM [56]. Recently, two spatially separated dynamic and phenotypically distinct CAF subtypes have been reported in pancreatic cancer named inflammatory CAFs (iCAFs) and myofibroblasts (myCAFs). The iCAFs with paracrine function, secreting cytokines and in particular IL-6, are reported located at a distance from tumor cells, whereas myCAFs depend on juxtacrine interaction with cancer cells and are able to remodel the stroma [57].

CAFs coevolve with the neoplastic cells during tumor progression by acquiring a pro-inflammatory gene expression pattern [58] and a consequence of this dynamic process may induce an EMT program in neoplastic cells. Indeed, the conditioned medium from NSCLC cells activates fibroblasts and increases their IL-6 production, which reciprocally induces EMT in cancer cells, resulting in chemoresistance [59]. The dynamic plasticity of tumor cells and CAFs has been elegantly reported by Del Pozo Martin and co-authors who have shown that mesenchymal cancer cells activate fibroblasts in an AXL-dependent EMT-activation *via* the secretion of Thrombospondin 2. In turn, in the site of metastatic colonization, the activated fibroblasts induce a switch toward a more epithelial proliferative phenotype in tumor cells, concomitant with an inhibition of TGF-β signaling [60]. Neoplastic cells drive the recruitment and activation of fibroblasts by secreting fibroblast-activating factors [56], TGF-β being the major player [61]. In agreement, TGF-β derived from tumor cells induces SNAIL expression in fibroblasts and its expression is essential to orchestrate an invasive program of breast epithelial cells [62].

Hence, the dialogue among tumor cells and the different cell components in the TME, mediated by soluble factors of inflammation, contributes in a dynamic fashion to regulate signaling pathways critical in the activation of EMT program.

ECM and mechanical cues.

A modified microenvironment characterized by inflammation and accompanied by tissue and matrix remodelling, the "reactive stroma", is frequent in tumors [63]. Tumor-associated stroma is enriched by a variety of ECM proteins, like collagens and proteoglycans, and glycoproteins, such as laminins and fibronectin and is continuously remodelled by MMPs, proteolytic enzymes that degrade cell-cell and cell-ECM adhesion molecules, permitting tumor invasion and metastasis [64].

It has recently been recognized that the mechanical properties of ECM govern tumor cell behaviour and integrate biochemical cues from the TME to regulate tumor progression and metastatic dissemination [65, 66]. Many ECM proteins contain binding sites for growth factors allowing the matrix to represent also a reservoir of soluble factors, as recently reported by Griggs and co-authors who elucidated a mechanism implicating fibronectin fibrillogenesis as responsible for TGF-β1-induced EMT signaling [67]. The latent form of TGF-β1 crosslinked to the ECM can be activated via a number of mechanisms including cellular force generation and integrin engagement both in epithelial cells and myofibroblasts (for an extensive review see [68]).

Mechanical properties of the TME are sensed by integrin receptors that connect ECM to the actin cytoskeleton inside the cells. Matrix stiffness generates integrin clusters, the assembly of focal adhesions and various intracellular signaling pathways related to actin cytoskeletal dynamics [69]. The alteration of the ECM-integrin axis leads to tissue architecture disorganization, characterized by cell-cell junction disruption and cell spreading resembling the EMT process [70].

An elegant paper from Radisky and colleagues reported that EMT may be activated by MMP-3 through the induction of the alternatively spliced isoform ras-related C3 botulinum toxin substrate 1 (RAC1)b, able to increase levels of reactive oxygen species (ROS), which in turn stimulate the expression of the transcription factor SNAIL [71]. Furthermore, in mammary epithelial cells cultured on stiff substrata or in collagen-rich regions, RAC1b localizes to the plasma membrane, where it promotes the production of ROS, expression of *Snail*, and activation of the EMT program [72], thus indicating that the mechanical properties of ECM affect transcription to direct tumor cell phenotype. In agreement, TWIST has been reported as a mechanomediator that promotes EMT in breast tumor cells in response to increased matrix stiffness. Mechanistically, increasing matrix stiffness induces integrin-dependent phosphorylation events allowing the nuclear localization of TWIST, EMT and invasion [73]. The specific requirement for ECM-mediated tumor cell invasion has been reported in lung adenocarcinoma where the CRK Like Proto-Oncogene (CRKL) adaptor

molecule was identified as a mediator of the ECM-dependent β1 integrin signaling and cancer cell invasion regulated by the microRNA-200/ZEB1 axis [74]. The functional characterization of the miR-200 targets indicates that miR-200 regulate cell invasion at multiple levels, including decreasing numbers of invadopodia, MMP activity, and focal adhesions [75]. Reciprocally, in mesenchymal lung cancer cells, the miR-200/ZEB1 axis regulates the lysyl oxidase 2 (LOXL2)–mediated collagen I and III cross-linking and deposition, driving lung cancer cell invasion and metastasis [76]. The importance of ECM remodelling and relevance of stiffness in inducing tumor progression is clearly shown by the finding that LOXL-mediated collagen cross-linking can modulate tissue fibrosis and stiffness to induce integrin-dependant premalignant breast epithelia invasion [77].

In response to elevated matrix stiffness the mechanosensitive transcription factors Yes associated protein 1 (YAP1, also known as YAP) and Tafazzin (TAZ) translocate to the nucleus of mesenchymal stem cells [78]. YAP is able to replace an oncogenic Kirsten rat sarcoma viral oncogene homolog (KRAS) signaling in lung cancer, by regulating an EMT-like transcriptional program [79]. The EMT-activator ZEB1 interacts with YAP and a common ZEB1/YAP target gene pattern, crucial for EMT-associated effects, has been reported as a strong predictor of poor clinical outcome in hormone receptor negative breast cancer [80].

The dynamics of ECM composition, organization and remodelling, as well as mechanical properties, represent biophysical cues able to influence the plasticity of tumor cells toward an EMT-related invasive behaviour.

Hypoxia and low pH.

A direct link between ECM composition/organization and hypoxia has been related to metastatic spread [81].

Based on recent reports, hypoxia, as a result of ischemic conditions, participates in EMT activation [14]. During disease progression, most tumors develop an aberrant vasculature network that tends to be poorly organized and leaky, disrupting blood flow, and hampering the delivery of oxygen and nutrients [82]. Hypoxia in turn, (*i*) leads to a metabolic rewiring towards anaerobic glycolysis that produces acidic metabolites (*i.e.*, lactic and carbonic acids), and (*ii*) hampers the ability of the microenvironment to remove tumor derived acids. Consequently, the pH of tumors is typically highly acidic [83]. Cellular adaptation to hypoxia and low pH underlies critical steps in tumor progression, altering the expression of genes encoding products required for increased oxygen delivery, anaerobic metabolism, angiogenesis, invasiveness, resistance to cancer therapy that synergize to induce EMT [82, 84, 85]. Specifically, *in vivo* xenografts of breast and colon cancers have given evidence of tumor cell-mediated accumulation of protons in the proximal TME, which becomes highly acidic. Such an acidic environment has been shown to exert negative effects on surrounding normal cells impelling local invasion [84]. Previous studies have showed that a low pH is a critical parameter of invasiveness and drug resistance in melanoma and prostate cancer models [85, 86]. Indeed, under hypoxic conditions, hypoxia-inducible factor (HIF)-1α, a transcriptional regulator of cellular responses to hypoxia, triggers the transcription of inducible EMT-related genes such as *TGF-β*, *TWIST* and *LOX* [87] as well as of other paracrine factors regulating tumor inflammation, angiogenesis and immunosuppression [88-90]. Hypoxia has been shown to induce, in ovarian carcinoma cells, E-cadherin downregulation via up-regulation of the transcriptional repressor SNAIL [91]. Moreover, HIFs work in concert with inflammatory cytokines to induce EMT [92, 93]. Accordingly, HIF-1α enhances *TWIST* expression and then EMT in rat models of prostate hyperplasia under inflammatory conditions (mainly mediated by IL-1β, IL-6, and TNF-α) [94]. Kou-Juey Wu and colleagues using a bioinformatic approach have identified histone deacetylase (HDAC)3 as a direct transcriptional target of HIF-1α. HIF1α-induced HDAC3 is essential for mesenchymal gene expression and also serves as an essential corepressor of epithelial genes [95]. Furthermore, hypoxia would drive ROS that are strictly related to cancer progression

[96] and able to induce *SNAIL* expression, shaping breast cancer cell morphology *in vitro* culture and tissue structure *in vivo*, leading to EMT-driven malignant transformation [71]. Despite the significant progress that has been made, much more work remains in order to identify and understand the complex and dynamic nets of hypoxia-related EMT programmes to be exploited for cancer care.

EMT and cancer immunoediting.

The immunoediting theory, originally developed to describe tumor–immune cell co-evolution, postulates the Darwinian immunoselection of tumor cell variants that escape T cell attack [97, 98]. Many works have documented that immunity can either prevent or control tumor outgrowth, or facilitate cellular transformation, shaping the immunogenicity of tumors [99].

Notably, one potential reprogramming mechanism activated during immunoediting is EMT. In 2006, Knutson KL *et al.*, showed that transplanting tumors from a neu-transgenic mouse model of breast cancer into syngeneic mice, stimulates a T cell dependent rejection. The immunoedited tumor cells were enriched in neu-negative variant cells and showed mesenchymal morphology [100]. Thus these authors have further demonstrated that CD8 T cells can stimulate mammary epithelial tumor cells to undergo EMT and to acquire a stem-like phenotype with increased tumorigenic capability and chemotherapeutic resistance [101]. Furthermore, in a mouse model of lung cancer a suppression of CD8 T cell function has been reported to be linked to EMT. The highly metastatic lung cancer cells have a low expression of miR-200 and the activation of miR-200/ZEB1 axis favours the EMT program, relieves programmed death-ligand 1 (PD-L)1 expression from miR-200 restraint, leading to CD8 T cell exhaustion crucial for metastasis promotion [102]. More recently a patient pan-cancer EMT signature across multiple tumor types showed a strong correlation of EMT markers with the immune checkpoint blockers (ICBs) PD-1, PD-L1, PD-L2 and cytotoxic T-lymphocyte antigen (CTLA)4 [103]. Lou and colleagues have associated lung adenocarcinomas showing mesenchymal

traits with an inflammatory TME, independent of tumor mutational burden and correlated with an increased expression of multiple targetable ICBs, suggesting a relationship between EMT and immune dysfunction and the need to further investigate EMT as a predictor of response to ICB therapy [104].

As a matter of fact, the interdependence of tumor cells, immune cells and TME closely connects the adaptive phenotypic plasticity during tumor progression. These reversible stochastic state transitions are a constant source of phenotypic heterogeneity, with important implications for resistance to diverse treatment modalities, including immunotherapy, chemotherapy and targeted therapies and are therefore a target of prime interest for oncological and immunological research.

EMT and cancer stem cells (CSCs).

Tumor progression is an evolutionary process in which subclonal populations of cells have a selective phenotypic advantage in a given TME context [105], characterized by an interconnected plasticity between tumor cells and their surrounding dynamic context. Thus, every moment, tumor composition is unique and highly heterogeneous. Tumor heterogeneity is commonplace between tumors from different patients (inter-tumor heterogeneity) and within a single tumor (intra-tumor heterogeneity). CSCs have been forwarded as one of the determining factors that contribute to intra-tumor heterogeneity and the root of aggressive, resilient tumors [106, 107]. Through genetic and epigenetic evolution, CSCs are able to differentiate into multiple tumor cell types sustaining the long-term clonal maintenance of the disease [106-108]. Accumulating experimental evidence suggests a *nexus* between EMT and CSCs. Indeed, EMT-promoting transcription factors (SNAIL, TWIST, and ZEB1) have recently been endowed with the power to confer self-renewal properties upon carcinoma cells [8]. First evidences of a link between EMT and CSCs come from experimental observations by Mani and colleagues, who showed that EMT inducers (*e.g.*, TGF-β1, SNAIL or TWIST) endow breast cancer cells with both a $CD44^{high}/CD24^{low}$ CSC expression patterns and enhanced migratory and invasive capabilities [109]. Likewise, TGF-β fuels the

proclivity of human basal breast non-CSCs to de-differentiate into CSCs depending on an epigenetic regulation of ZEB1 [110]. As such, in skin squamous cell carcinoma, TGF-β signaling bestows CSC proliferation, tumor heterogeneity and drug resistance [111]. Likewise, SLUG has been recently added to the list of these EMT-related transcriptional factors inducing stem traits [112]. The combined action of SLUG and SRY-box 9 (SOX9) has been reported to confer stem-like properties of mammary and lung cells [113]. Therefore, TWIST cooperates with BMI1 proto-oncogene, polycomb ring finger (BMI1) to promote EMT and the tumor-initiating capability leading to a poor prognosis of head and neck cancer patients [114].

Canonical WNT activity is highly related to cancer stemness [115] and EMT promotion [116]; furthermore, both the mesenchymal state and CSC phenotype need a continual autocrine/paracrine signaling.

All this experimental evidence suggest that the activation of the EMT program places neoplastic epithelial cells in states where they are poised to enter into stem cell compartments.

The link between EMT and CSCs is highly related to the microenvironmental molecular context and different layers of complexity indicate that tumor stemness and EMT can be regulated by distinct mechanisms, but this goes beyond the scope of this review.

Antitumor drugs and EMT induction.

Development of resistance to anticancer treatment continues to be a major impediment in medical oncology. Cancer therapy resistance, may not only precede, but also could arise as a result of therapy. Doxorubicin, a cytotoxic drug commonly used in clinical practice to treat solid and haematological malignancies, may promote resistance in HCT 116 colon cancer cells, in part via the activation of TGF-β signaling triggering SNAIL, SLUG, vimentin and N-cadherin expression [117]. Highlighting the ambivalent effect of chemotherapeutic agents, Bruchard *et al*., reported that gemcitabine and 5-fluorouracil cause lysosomal release of cathepsin B from tumor cells, which

activates the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome in MDSCs. MDSCs then, produce IL-1β that leads to IL-17 secretion by T cells and promotes tumor growth and chemotherapy resistance [118]. Accordingly, murine and human breast cancer cells respond to chemotherapy stress through the acquisition of a skill set of cytokines that allow them to survive and metastasize. Indeed, hit cancer cells release the chemokine CXCL1 and CXCL2 triggering a paracrine network that mediates lung metastasis and chemoresistance [116]. To the same extent, radiation-, anti-angiogenic- and immune-therapy can promote malignancy and EMT traits. NSCLC cells that have survived irradiation have been shown to have a complex phenotype including all the features of epithelial-mesenchymal transitioned CSCs [119]. Recently, Aguilera *et al.*, showed that in PyMT tumor cell lines the receptor tyrosine kinase AXL is a main predictor of radiation therapy resistance. AXL overexpression in unresponsive tumors induced the downregulation of the major histocompatibility complex (MHC)-I and the promotion of a suppressive TME [120]. Interestingly, AXL has gained much attention as it has shown to be induced by HIF-α and hypoxia and to be a mediator of tumor cell invasiveness [121, 122] and a regulator of EMT [123]. Likewise, anti-VEGF treatment has been reported to trigger tumor cells to release proinflammatory signals, which in turn act in a paracrine and autocrine manner to establish an immunosuppressive environment and to induce EMT, respectively [124, 125]. Recently Hugo and colleagues associated innate resistance of anti-PD-1 therapy in melanomas with the upregulation of EMT-related genes, as well as of genes involved in ECM remodelling, cell adhesion, angiogenesis and wound healing [126]. Similarly, previous data from Boisgerault *et al.*, suggested that immunotherapeutic vaccinations to induce T cell responses, could place a selective pressure on tumor cells favouring the appearance of CSCs or cells undergoing EMT [127].

It is worth noting that therapy-induced resistance and EMT are still poorly understood processes. Given the complexity and the heterogeneity of EMT-related effects, further comprehensive studies

are needed to untangle how the EMT program contributes to the development of resistance to different therapeutic treatments in order to achieve more effective combined anti-cancer strategies.

Concluding remarks.

The mechanistic connections between ontogeny and tumor pathogenesis have opened new interest in EMT research and laid the ground for its therapeutic manipulation.

However, given the complex, intertwined circuitry regulating EMT during cancer progression, it would be deemed necessary to decipher the TME-related loop which contributes to the onset and the maintenance of EMT. Indeed, as is apparent from this overview, the EMT program cannot be efficiently induced by activation of one or another signaling pathway, but rather by the cooperation and convergence of multiple pathways on common targets, reactivated after the loss of organ architecture [128]. The identification of the mechanistic determinants involved in the association of EMT with immune suppression needs future studies, and as summarized in this review these processes may likely occur through several mechanisms. We can foresee that EMT may contribute to resistance to the new immunotherapeutic treatments, a promising reality in cancer treatment. Future work will be required to identify reliable EMT markers in tumors to define the EMT as predictive of response to immune stimulatory therapies.

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Legends to figures.

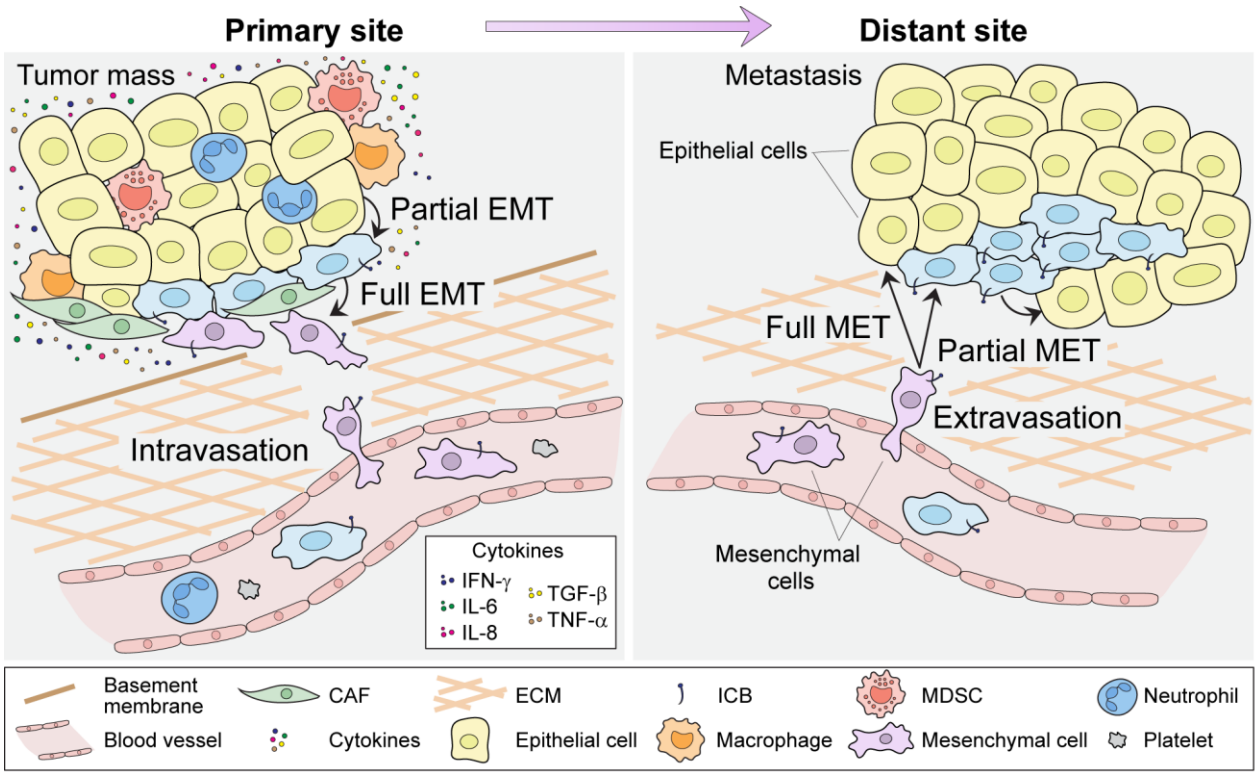

Figure 1

Figure 1: Complex networks orchestrating EMT.

Soluble inflammatory mediators (IFN-γ, IL-6, IL-8, TNF-α and TGF-β), immune infiltrating macrophages, MDSCs, neutrophils, platelets and CAFs can promote an EMT program in primary tumors. Mesenchymal cells are then able to invade the surrounding stroma and eventually enter the systemic circulation. Once circulating tumor cells reach distant sites, undergo a MET program that is crucial for the outgrowth of metastases. CAF, cancer-associated fibroblast; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; ICB, immune checkpoint blocker; IFN, interferon; IL, interleukin; MDSC, myeloid-derived suppressor cell; MET, mesenchymal-epithelial transition; TGF-β, tumor growth factor-β; TNF-α, tumor necrosis factor-α.

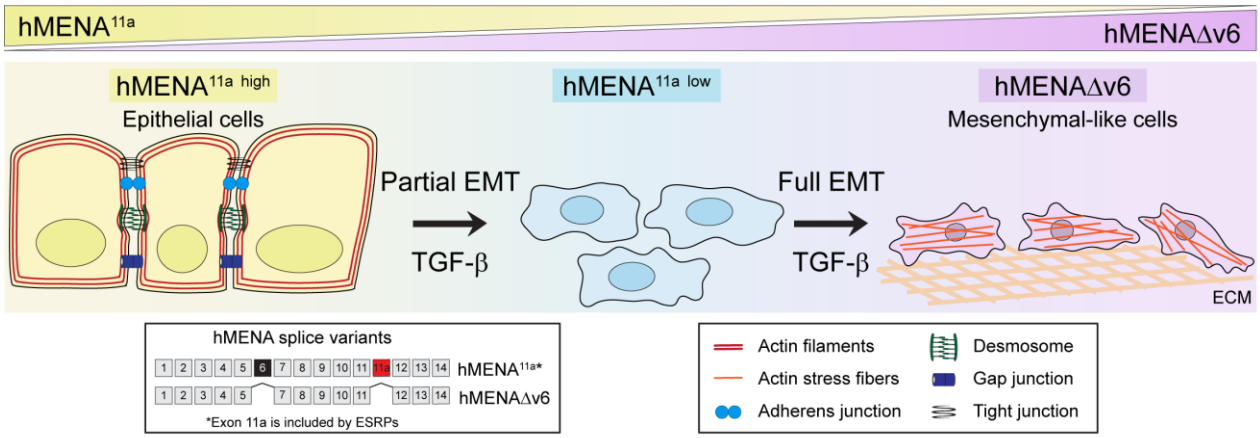

Figure 2

Figure 2: hMENA alternative splicing and EMT.

hMENA$^{11a\ high}$ are epithelial tumor cells interconnected through desmosomes, tight, gap and adherens junctions. TGF-β signaling induces a shift toward a mesenchymal phenotype accompanied and sustained by the down-regulation of hMENA^{11a} and the up-regulation of the pro-invasive hMENAΔv6 isoform. ECM, extracellular matrix; EMT, epithelial–mesenchymal transition; ESRP, epithelial splicing regulatory protein; TGF-β, tumor growth factor-β.

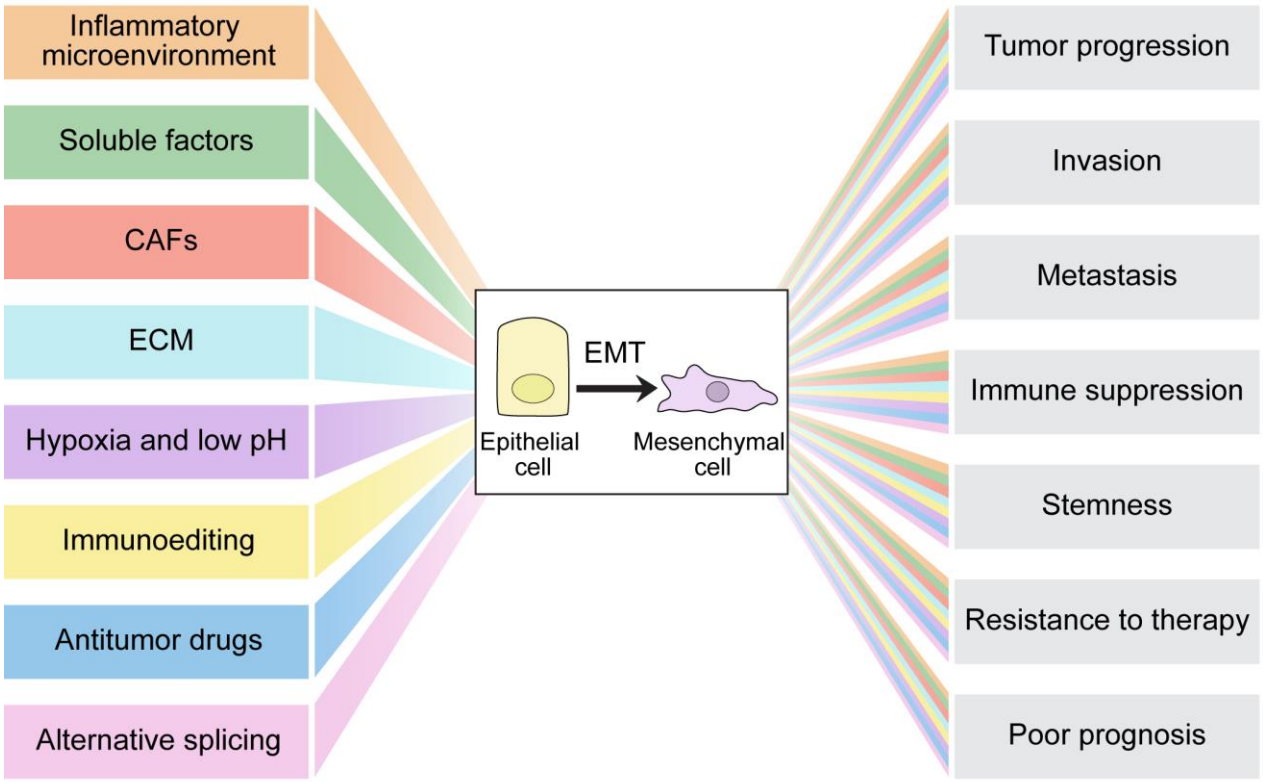

Figure 3: *Stimuli*-responses during EMT.

Different *stimuli* induce an EMT program in tumor cells related with the acquisition of invasiveness, metastatic potential, immune escape abilities, stemness and resistance to therapy finally favouring tumor progression and correlating with poor prognosis. CAF, cancer-associated fibroblast; ECM, extracellular matrix; EMT, epithelial–mesenchymal transition.

Table 1. Morphological and functional changes during EMT.

Feature	Epithelial Cells	Mesenchymal Cells
Morphological traits	Polygonal and cobble-stone like	Elongated and spindle-like
Polarity	Apical-basal	Front-back
Cell adhesions	Desmosomes, adherens, gap and tight junctions with adjacent epithelial cells	Focal adhesions with the ECM
Motility	Non-motile	Motile and invasive
Selected markers	E-cadherin, cytokeratins, ESRPs, hMENA[11a]	N-cadherin, fibronectin, vimentin, FSP-1, α-SMA, hMENA Δv6, MMPs, SMAD, SNAIL, SLUG, TWIST, ZEB1

Abbreviations: EMT, epithelial–mesenchymal transition; ESRPs, epithelial splicing regulatory proteins; MMP, metalloproteinase; SLUG, snail family transcriptional repressor 2; α-SMA, α-smooth muscle actin; SMAD2, SMAD family member 2; SNAIL, snail family transcriptional repressor 1; TWIST, twist family bHLH transcription factor 1; ZEB1, zinc finger E-box-binding homeobox-1.

Table 2. Common *stimuli* and crosstalks in EMT and tumor invasiveness.

Stimulus	Subgroup	EMT relation	References
Inflammatory microenvironment	TAMs	• TGF-β production • Stabilization of SNAIL via TNF-α • EGF production	[28]; [29] [30] [31]
	MDSCs	• EGF, HGF and TGF-β production	[34]
	Neutrophils	• Activation of the CXCL8-CXCR2 axis	[33]
	Platelets	• TGF-β production	[35]
Soluble factors	TGF-β	• Activation of SMAD pathway, miRNA expression, activation of mTORC1 pathway, regulation of RHO-GTPases • ESRP repression by TGF-β/ZEB1 • TGF-β-mediated hMENAΔv6 induction and hMENA^{11a} down-regulation	[37]; [38]; [39] [17]; [21] [21]
	IL-6	• JAK-STAT3-mediated *SNAIL* expression • E-cadherin downregulation and induction of Vimentin, N-cadherin, SNAIL AND TWIST • Positive feedback loop with STAT3 and miR-34a	[10] [41] [42]
	TNF-α	• SNAIL and β-catenin stabilization via NF-κB and AKT • Cooperation with IL-1β • Cooperation with IFN-γ	[11] [45] [46]
	IL-8	• Overexpession of *BRACHYURY*	[49]
	C3 protein	• E-cadherin downregulation	[54]
	C1q	• Enhancement of fibronectin functions	[55]
CAFs		• Acquisition of a pro-inflammatory gene expression pattern • Positive feedback loop with TGF-β produced by cancer cells	[57] [60]; [61]
ECM and mechanical cues	ECM	• Fibronectin fibrillogenesis • Matrix stiffness • Alteration of cytoskeleton • Snail expression by MMP-3-induced ROS • Activation of the adaptor molecule CRKL	[67] [68]; [69]; [73]; [77] [69] [71]; [72] [74]
	Mechanosensitive transcription factors	• Matrix stiffness • Cooperation with ZEB1 • TWIST nuclear localization	[78]; [79] [80] [73]
Hypoxia and low pH	HIF-1	• Upregulation of TGF-β, TWIST and LOX • Upregulation of paracrine factors • E-cadherin downregulation via SNAIL • Cooperation with inflammatory cytokines • HDAC3-mediated repression of epithelial genes • ROS-mediated *SNAIL* expression	[87]; [94] [88]; [89]; [90] [91] [92]; [93]; [94] [95] [71]
Immunoediting		• Upregulation of mesenchymal genes and traits • Activation of miR-200/ZEB-1 axis	[100]; [101] [102]
Antitumor drugs	Doxorubicin	• TGF-β, vimentin, N–cadherin, SNAIL and SLUG upregulation; TNF-α, CXCL1 and CXCL2 release	[116]; [117]
	Gemcitabine and 5-fluorouracil	• IL-1β release from MDSCs and IL-17 release from T cells	[118]
	Radiation therapy	• SNAIL, vimentin, N-cadherin upregulation • AXL overexpression and MHC-I downregulation via	[119] [120]; [123]

		HIF-α	
Anti-VEGF	•	E-cadherin downregulation, vimentin expression	[124]; [125]
Anti-PD-1	•	TWIST, LOX upregulation	[126]
Vaccines	•	E-cadherin downregulation, N-cadherin upregulation	[127]

Abbreviations: AKT, AKT serine/threonine kinase 1; AXL, AXL receptor tyrosine kinase; CRKL, CRK Like Proto-Oncogene; CRTC1/TORC1, CREB regulated transcription coactivator 1; CXCL, C-X-C motif ligand; CXCR, C-X-C chemokine receptor; ECM, extracellular matrix; EGF, epidermal growth factor: EMT, epithelial–mesenchymal transition; ESRP, epithelial splicing regulatory protein; HDAC3, histone deacetylase 3; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible factor-1α; IL, interleukin; JAK, Janus kinase; LOXL2, lysyl oxidase 2; MDSC, myeloid-derived suppressor cell; MHC-I, major histocompatibility complex-I; MMP, metalloproteinase; NF-κB, nuclear factor-*κ*B; PD-1, programmed death 1; ROS, reactive oxygen species; RHO, rhodopsin; SLUG, snail family transcriptional repressor 2; α-SMA, α-smooth muscle actin; SMAD2, SMAD family member 2; SNAIL, snail family transcriptional repressor 1; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage; TGF-β, tumor growth factor-β; TNF-α, tumor necrosis factor-α; TWIST, twist family bHLH transcription factor 1; VEGF, vascular endothelial growth factor; ZEB1, zinc finger E-box-binding homeobox-1.