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# Epithelial-to-mesenchymal transition as the driver of changing carcinoma and glioblastoma microenvironment☆

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ABSTRACT

Epithelial-to-mesenchymal transition (EMT) is an essential molecular and cellular process that is part of normal embryogenesis and wound healing, and also has a ubiquitous role in various types of carcinoma and glioblastoma. EMT is activated and regulated by specific microenvironmental endogenous triggers and a complex network of signalling pathways. These mostly include epigenetic events that affect protein translation-controlling factors and proteases, altogether orchestrated by the switching on and off of oncogenes and tumour-suppressor genes in cancer cells. The hallmark of cancer-linked EMT is that the process is incomplete, as it is opposed by the reverse process of mesenchymal-to-epithelial transition, which results in a hybrid epithelial/mesenchymal phenotype that shows notable cell plasticity. This is a characteristic of cancer stem cells (CSCs), and it is of the utmost importance in their niche microenvironment, where it governs CSC migratory and invasive properties, thereby creating metastatic CSCs. These cells have high resistance to therapeutic treatments, in particular in glioblastoma.

## 1. Introduction

The epithelium is one of the basic tissue types in animals and it consists of one or more layers of differentiated cells that are attached to the basement membrane *via* hemidesmosomes. Epithelial cells show static apical–basal polarity and are connected to each other laterally through tight gaps, adherens junctions and desmosomes. Certain triggers associated with more complex processes, such as ontogenesis, tissue regeneration and cancers, can induce epithelial-to-mesenchymal transition (EMT). This is a programme that results in morphological and functional transformation of the epithelial phenotype. In cancers, this programme imparts heritable phenotypic changes to carcinoma cells through epigenetic modifications, without introducing new genetic alterations. In this way, epithelial cells lose their apical–basal orientation and switch to a more migratory, spindle-like shape, with front–rear cell polarisation.

Epithelial-to-mesenchymal transition is a reversible cell programme, and the mesenchymal phenotype that results can regain epithelial cell characteristics in the process termed mesenchymal-to-epithelial transition (MET). The connections among cells break up when they progress towards the mesenchymal state, and the basement membrane and cytoskeleton become reorganised. As well as a greater migratory ability, mesenchymal traits include enhanced cell invasion, which involves the degradation of their own extracellular matrix (ECM) with ECM-degrading enzymes, in addition to acquired resistance to apoptosis. This whole process is triggered epigenetically and controlled by EMT-inducing transcriptional factors (EMT-TFs) that act in different combinations, and result in altered expression of the genes that control cell transition [1–3]. Epithelial cells initially express proteins that help to maintain their typical polarity, with the most representative being cadherin (E-cadherin) and some other epithelial cell adhesion proteins, such as occludins, claudins, various β-integrins and cytokeratins, which

*Abbreviations:* CSCs, cancer stem cells; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; GB, glioblastoma; GSCs, glioblastoma stem cells; MSCs, mesenchymal stem cells; TFs, transcription factors

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are required for the structural integrity of epithelial tissues. These are generally down-regulated in the mesenchymal cell type due to epigenetically modified expression of selective TFs of ZEB, SNAIL, SLUG and the TWIST family, which induces silencing through hypermethylation and histone deacetylation [4]. On the other hand, these TFs enhance the expression of genes associated with the mesenchymal state, such as N-cadherin, vimentin, fibronectin and the β-integrins, as well as the ECM-degrading proteases [5].

However, not all these molecules are involved in a particular epithelial or mesenchymal cell type. Molecular variations in EMT have been observed in normal and cancerous cells. Moreover, epigenetic cues are not only activated endogenously, but more commonly they originate from the cell microenvironment, which might induce EMT-TFs in various combinations in an epithelial cell. Thus, EMT should be understood only in terms of the principle that cell phenotypes can constantly, and even reversibly, change. Even if the resulting phenotypes have a temporary existence, they drastically change their function as a result of cell–cell cross-talk in the altered tumour microenvironment. These complex communications are either juxtacrine or paracrine, and they are mediated by secreted chemokines and growth factors, gap-junctions, nanotubules or extracellular vesicles [6]. For example, cancer-derived exosomes have been shown to promote increased cellular aggressiveness, as increased cell motility, migration and invasion. Exosomes can function to concentrate proteins or RNA for signalling and transformation of nearby cells, by alteration of the tumour microenvironment as a so-called 'field effect' [7] and by pre-metastatic niche formation [8]. Cancer-cell-derived exosomes have been demonstrated to induce EMT *via* the expression of mesenchymal markers in recipient cells, which contributes to the progression to a more aggressive phenotype [6]. Signals that trigger EMT in cancer come from the surrounding stroma as well as from the stromal conditions, such as low levels of oxygen, cytokines and growth factors secreted into the tumour microenvironment, alternations in metabolism of neoplastic cells, and even by anti-tumour drugs.

In addition to multiple possible pathways that govern EMT-like processes, the second emerging concept from numerous studies (reviewed by Roche et al. [9]; Jolly et al. [10]; Dongre and Weinberg [1]) is that the EMT programme can generate various stable phenotypic states along the epithelial–mesenchymal spectrum that have features of both epithelial and mesenchymal cells. Thus, EMT can be interrupted before completion, which will give rise to various intermediate hybrid epithelial and mesenchymal phenotypes without acquisition of the complete mesenchymal traits [11,12]. This might be due in part to the simultaneous occurrence of the reversible MET process, which will result in an equilibrium where the precise molecular context remains unknown. Such mixed populations of cells can migrate together in cohorts that can reach higher levels of aggressiveness than fully transitioned mesenchymal cells. As addressed by Dongre and Weinberg [1] in studies where certain EMT-TFs were experimentally expressed at high constitutive levels, complete EMT can be achieved, although this might be a rare case under physiological conditions in wound healing, and in some cases during carcinoma progression.

There are three types of EMT known. Type 1 EMT is crucial during early development of an organism, and it occurs early after fertilisation. It is associated with early gastrulation, mesodermal development and endocardium morphogenesis, each of which has its own pattern of genetic control. Type 2 EMT relates to traumatic events like injuries and pathogen infections that lead to inflammation, which are mediated by inflammatory cells. Following tissue damage, fibroblasts and inflammatory cells infiltrate the tissue to establish homeostasis, accompanied by mesenchymal stem cells (MSCs). Type 3 EMT is associated with cancer progression, as described in this review.

## 2. Epithelial-to-mesenchymal transition as a key process in carcinomas

### 2.1. Cancer invasion in the metastatic tumour microenvironment

Cancer cells emerge through the transformation of normal cells into neoplastic cells *via* two consecutive or simultaneous processes: initiation and promotion. These can result in clonal expansion and development of benign tumours, which can evolve progressively to a malignant state. Cancer cells acquire a succession of characteristics, which are termed the hallmarks of cancer [13]. These include sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, and activation of invasion and metastasis formation. At the molecular level, during their clonal expansion, tumour cells acquire inherent genetic modifications that increase the stability of their genome or chromosomes. Exogenous triggers provide mutual interactions of cancer cells with the local tumour microenvironment, which includes inflammatory conditions. Alternatively, these triggers result from systemic immune responses, which enable the overall spread of a cancer. The final stage is then metastasis formation, through dissemination of evolutionary selected clones, which then colonise distant anatomical sites. Contrary to the ongoing search for patterns of genetic changes associated with each type of cancer, identification of the relatively small sets of up-regulated oncogenes and down-regulated tumour-suppressor genes has been shown to be the key switch for the expression of metastatic hallmarks [1]. Moreover, this suggests that the invasion–metastasis cascade is to a large extent initiated by epigenetic EMT programmes that have been recently recognised as essential, in particular for carcinomas that originate from epithelial cells. However, formation of micrometastases at secondary sites might not necessarily be followed by colonisation of the secondary organ, where the new tumour microenvironment will dictate the EMT/MET balance in the micrometastases.

Even more complexity is added to metastatic progression by the primary tumours that are comprised of multiple genetically distinct sub-clones. Metastatic cells that develop within these heterogeneous subsets of cells can evolve further into behaviourally and genetically distinct cell clusters [14]. Independent genetic evolution of metastatic clones is driven by the changing tumour microenvironment, particularly in the secondary organ, and it is therefore superimposed on the existing genetic heterogeneity of the primary cancer. The decisive selection of the most efficient seeding clone at the distant secondary site can be considered as Darwinian-like selection, where the tumour microenvironment in the metastatic niche has a crucial role. The relevance of the host organ tumour microenvironment in patients is shown by the metastases within any given secondary organ, as these are genetically more similar than metastases in other organs. This suggests the importance of adaptation of metastatic clones to the organ-specific microenvironment, which was reviewed by Lah et al. recently [15]. When invading cells reach their destination, they resemble the original epithelial carcinoma cells, although they do not have a mesenchymal appearance, as they reverse through MET at the secondary site, to remain there as silent metastases. To colonise the organ and metastasise (*i.e.* form metastases), cancer cells reverse EMT programme *via* silencing of various EMT-TFs at secondary organ parenchyma, or could involve the active, still uncharacterized proteins that repress EMT-TF expression. This can then promote transition of the cells towards a more migratory state, and the consequent organ colonisation [16].

#### 2.1.1. Molecular EMT triggers and inhibitors

Epithelial-to-mesenchymal transition consists of a balance of a network of proteins, which include epithelial E-cadherin, mesenchymal N-cadherin, vimentin, cell cytoskeleton polarity complexes and

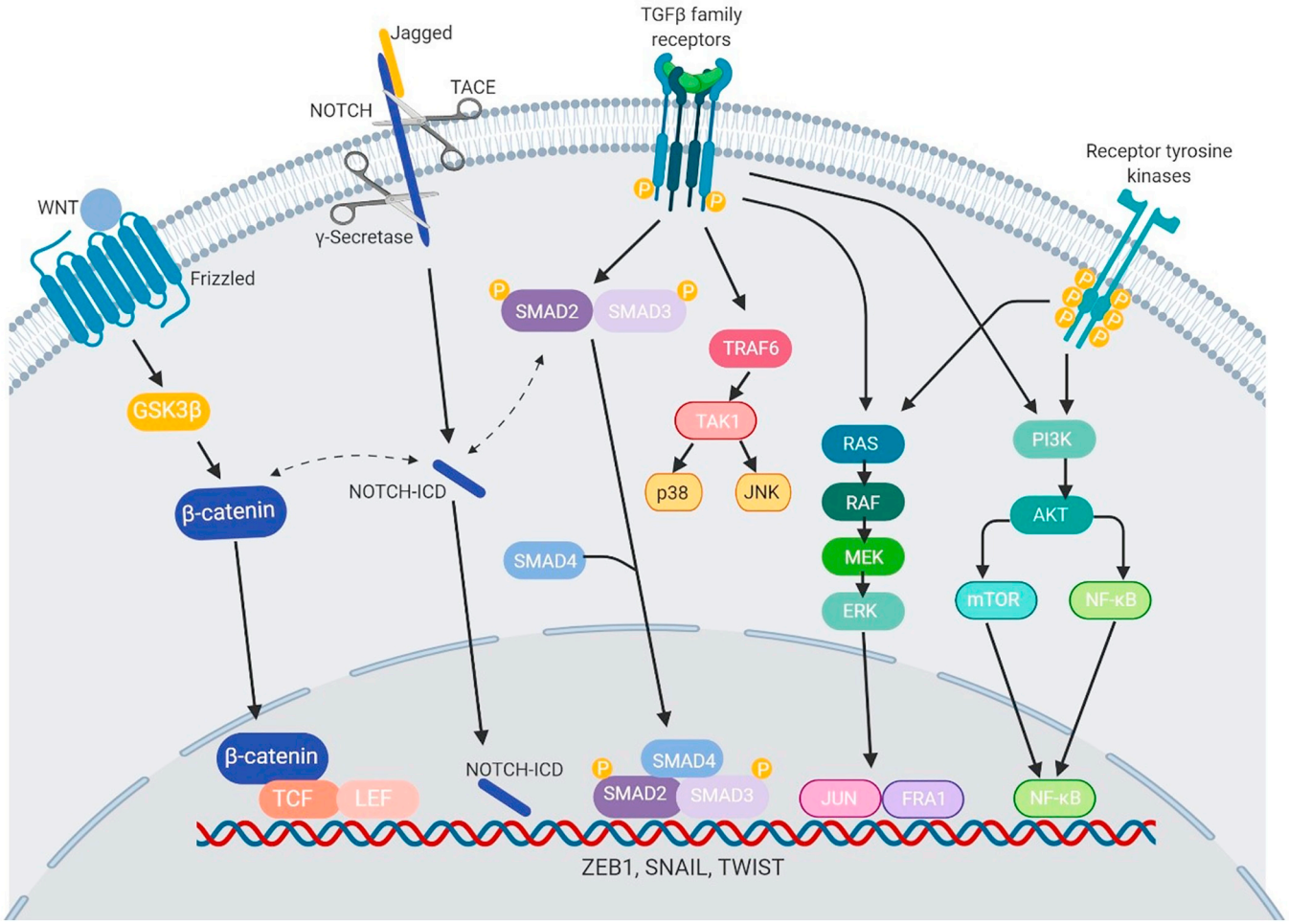


**Fig. 1.** Major EMT-signalling pathways.
Epithelial-to-mesenchymal transition (EMT) is regulated by shared WNT, NOTCH, TGFβ and tyrosine kinase receptor (TRK) signalling pathways that can induce activation of the ZEB1, SNAIL and TWIST transcription factors. These factors promote EMT by repression of epithelial marker genes and activation of genes characteristic for the mesenchymal phenotype. The WNT pathway is activated by the binding of a WNT-protein ligand to a Frizzled family receptor, which inhibits glycogen synthase kinase-3β (GSK3β) to stabilise β-catenin against cytosolic proteasome degradation. β-catenin then translocates to the nucleus and binds to the transcription factors TCF (T cell factor) and LEF (lymphoid enhancer-binding factor) to activate EMT-related gene expression. NOTCH signalling is initiated when Delta-like or Jagged family of ligands binds to the NOTCH receptor, which triggers a cascade of proteolytic cleavage by tumour necrosis factor-α-converting enzyme (TACE) and γ-secretase, which results in the release of the intracellular domain of the NOTCH receptor. This latter enters the nucleus and activates SNAIL2 expression. EMT is also mediated by TGFβ canonical (SMAD-dependent) and non-canonical (SMAD-independent) pathways. Upon ligand binding, the TGFβ family of receptors are phosphorylated, which leads to phosphorylation of the SMAD2 and SMAD3 proteins and binding of SMAD4 to the complex. This complex is then translocated to the nucleus, which leads to activation of EMT-associated transcription factors. In addition, TGFβ can activate the PI3K-AKT, RAS-RAF-MEK-ERK, p38 MAPK and JNK pathways. Different growth factors, including epidermal growth factor (EGF), can induce EMT through receptor tyrosine kinases (RTKs), which activates the RAS-RAF-MEK-ERK and PI3K signalling cascades that favour the mesenchymal phenotype. These pathways can be triggered at the same time and may be cross-linked, which results in activation of the EMT programme.

proteases. This balance is affected by inputs from a variety of signalling pathways, including those of transforming growth factor β (TGF-β), receptor tyrosine kinases (RTKs), multiple p53 pathways and hypoxia-induced factor (HIF-1α). These can induce the transcription of signalling that involves the immune- and inflammatory-related TF NF-κB, such as Wnt and Notch (Fig. 1). These pathways can be divided into two types in terms of their effects on a cell phenotype: they either induce migration and invasiveness, as the result of EMT, or they promote stemness characteristics. However, these two effects are not mutually exclusive.

The invasive phenotype is mainly enhanced by the following mediators/inducers of these pathways:

TGF-β is a member of a large family of proteins that have effects on cell differentiation and proliferation, and immune responses. TGF-β controls transcription by orchestrating non-coding RNAs, which depends on cell type and context [17]. EMT is mediated by TGF-β signalling through both canonical (SMAD dependent) and non-canonical (SMAD independent) pathways [18]. Binding of a number of these TGF-β family protein ligands to their receptor type II (TβRII) leads to its dimerization with TβRI, followed by clustering of SMAD proteins in the nucleus, which then cooperate with other TFs (Fig. 1). TGF-β signalling has also been suggested to have a crucial role in several features of cancer stem cells (CSCs) [19] (see Section 3). In addition, TGF-β regulates EMT through binding microRNA miR-200 and promoting the actions of long non-coding RNAs [1,10].

MicroRNAs such as the miR-200 family are involved as EMT modulators, while they can also target some stemness markers, including *SOX2* and *KLF4* [11]. Forte et al. [11] further stated that in stem cells and cancer cells, ZEB1 inversely modulates EMT by down-regulation of the miR-200 family, which induces stemness-related TFs. Reciprocally, the miR-200 family can regulate EMT through inhibition of *ZEB1* and *ZEB2*, two known E-cadherin repressors. Decreased miR-200c levels are

associated with promotion of EMT and a concomitant increase in the abundance of mammary epithelial stem cells [20].

Membrane RTKs also have roles in EMT-induced cell invasion (Fig. 1). Among these, the epidermal growth factor receptors (EGFRs) are most commonly activated in many types of cancers. EGF ligand homologues can bind to the EGFR extracellular domain to trigger a series of EMT-linked events, the most notable being activation of the PI3K/Akt, Ras–Raf–MEK–MAPK, JAK/STAT and MEK/ERK cancer-promoting signalling pathways. Also RTKs such as fibroblast growth factor receptor, platelet-derived growth factor receptor, keratinocyte growth factor receptor, hepatocyte growth factor receptor (cMET) and others, can activate EGFR indirectly or directly *via* cross-linked signalling regulation and expression of TFs, like ZEB1/2 and SNAIL1/2, E47 and TWIST [21,22] (Fig. 1). Of note, the EGFR and platelet-derived growth factor receptor are highly expressed in glioblastoma (GB), and represent markers of GB subtypes with significantly different phenotypes [23].

Transcription factors ZEB1 and ZEB2 are among the most important TFs that drive EMT, and they show some overlapping effects [24]. They promote EMT by repression of the epithelial state and activation of the mesenchymal state. They act through regulation of the activity of histone deacetylases, histone methyltransferases, Polycomb and coREST. The cell phenotype is controlled in part by a ZEB/miR-200 double-negative loop: while miR-200 represses both *ZEB1* and *ZEB2*, in turn, they repress miR-200 transcription, thereby stabilizing the epithelial or mesenchymal equilibrium states [25]. Both ZEB1 and ZEB2 are also controlled by SNAIL and TWIST [26].

Transcription factors SNAIL1, SNAIL2 and SNAIL3 target genes that influence histone post-translational modifications, thus repressing E-cadherin and inducing mesenchymal markers. Notch signalling enables and stabilizes *SNAIL* gene expression during HIF-1α–induced hypoxia (see below) [10].

TWIST1 and TWIST2 bind to DNA through their basic/helix-loop-helix domain, which also mediates their oligomerization. Depending on the binding partners, the TWISTs can have vastly different effects. For instance, they can promote transcription of N-cadherin and repression of E-cadherin. TWIST can promote EMT *via* activation of the above-described TGF-β/SMAD cascade, and TWIST can in turn be activated by TGF-β signalling, as well as by Wnt, hypoxia (HIF-1α), inflammatory signals, and some RTKs [26,27].

### 2.1.2. The roles of cell stemness markers and EMT signalling pathways

Four major signalling pathways have been demonstrated as common to normal stem cells and cancer cells; *i.e.*, the Notch, Wnt, Hedgehog and Bmp-1 pathways [13]. The first two of these have been more commonly described in association with EMT, where they induce stemness characteristics in cancer cells.

The Notch group of signalling proteins comprises four transmembrane receptors that are bound by two types of ligands: Delta (DLL1, 3, 4) and/or Jagged (JAG1, 2), which are present on the neighbouring cells to cancer cells (Fig. 1). Notch has an important role in embryogenesis-related EMT [1], whereas Notch signalling is enhanced in nearly all carcinomas [28]. Notch signalling can induce EMT and maintain stemness; however, our understanding of the different roles of these two sub-families of ligands of Notch signalling (*i.e.*, Delta, Jagged) is still incomplete in the context of EMT and cancer stemness [29]. A link to the 'EMT circuit' was defined by Jolly et al. [10], *w*here the Notch intracellular domain can activate *SNAIL* to promote EMT when the cells are coupled to Jagged, but not to the Delta ligand. Due to this lateral induction mechanism observed in Notch–Jagged signalling, clusters of cancer cells that interact *via* Notch–Jagged signalling can mutually stabilise their 'metastable' phenotype, which is in an EMT–MET equilibrium state, to thus maintain high 'stemness' potential. Such cells have been seen to be more metastatic, with additional colonisation potential [28]. Jagged1 is thus emerging as a potential therapeutic target due its role in maintaining CSCs [30].

The Wnt family is a group of 19 glycoproteins in humans that can bind to the extracellular domain of the Frizzled receptors, a family of G-protein-coupled receptors (Fig. 1). The Wnt proteins bind to Frizzled in cooperation with their co-receptors, which results in activation of the canonical Wnt/β-catenin pathway or the non-canonical Wnt/$Ca^{2+}$ pathway. Physiological Wnt-signalling-mediated EMT is required in embryonal neural development, cell differentiation, proliferation and motility, and wound healing. However, it is also important in several cancers [31].

Activation of Wnt signalling releases β-catenin, which then translocates into the nucleus to act as a transcription co-factor to induce expression of the TWIST, SNAIL and ZEB1 TFs. As mentioned above, β-catenin is also a component of adherens junctions that in the epithelial cell state binds all of the free cytosolic β-catenin. EMT-induced disruption of these junctions allows for β-catenin nuclear translocation and signalling. Independently, in pancreatic cancer cells, this β-catenin signalling has been shown to activate miR-300 and miR-136 [32], whereas miR-23a regulates canonical Wnt signalling in breast cancer cells [33].

Collectively, these various key players in EMT can be activated independently and/or in the above described pathways, to interact with each other at numerous points. These synergising EMT pathways are shown in Fig. 1.

The core regulatory network for EMT/MET-like processes acts as a 'three-way switch', to give rise to three distinct phenotypes: the epithelial phenotype, the mesenchymal phenotype that results from completed EMT, and a hybrid E/M phenotype. This represents the theoretical framework to validate and understand the roles of the many players in the regulation of epithelial plasticity. To define this equilibrium, an 'EMT score' was constructed. Due to the EMT/MET balance, clones of the intermediate phenotype acquire the metastable phenotype with high plasticity, which defines the CSC characteristics (see Section 3.). These clones can convert between each other, dependent upon the cues provided by the tumour microenvironment. Furthermore, we can highlight recent studies on the impact of partial EMT on cell migration and the formation of clusters of metastasising cells, which are also known as circulating tumour cells.

### 2.1.3. Molecular basis of EMT-enhanced cell invasion

It is not entirely clear how cancer cells gain their migratory phenotype, although we aim here to define the key players that lead to the increased cell invasiveness that we ascribe to EMT. Cell invasion comprises three major consecutive steps: detachment from the primary tumour; ECM degradation; and cell migration. First, the loss of homotypic cell adhesion in carcinomas requires down-regulation of E-cadherin in the cells. An extensive network of signalling pathways steers this process, which is initiated by selective TFs, such as SNAIL, ZEB, TWIST and E12/E47, which have important roles in the many steps to cancer progression, and in particular in cell invasion, dissemination and metastasis formation in the colonisation of secondary tissues [34]. In addition to some others, TFs like Brachyury, Goosecoid, SIX1 and PRRX1 can directly or indirectly repress E-cadherin, which is the hallmark of the epithelial phenotype [5]. As reviewed by Jolly and co-workers [10], these can be induced by epigenetic changes, silencing, post-translational modifications, alternative splicing and changes in chromatin. The epithelial phenotype corresponds to high levels of miR-200 and miR-34, whereas the mesenchymal phenotype corresponds to high levels of ZEB and SNAIL. These components form two interlinked mutually inhibitory feedback loops, as miR-34/SNAIL and miR-200/ZEB, such that other EMT-inducing signals from TGF-β, EGF, HGF, and Notch can activate ZEB and SNAIL, whereas p53 activates miR-200 and miR-34 [5]. In many carcinomas, these signals converge on the core EMT regulatory network, which is also referred to as the 'motor of cellular plasticity', due to its coupling with many other cellular processes as well as cell invasion, such as apoptosis, the cell cycle, metabolism and immunosuppression.

As indicated above, the initial event in epithelial cells upon triggering of EMT is the loss of plasma-membrane-associated E-cadherin. As well as being epigenetically down-regulated, the loss of this cell-cell homotypic adhesion protein is due to enhanced endocytosis and its subsequent degradation in lysosomes [18]. As a result, the co-transcription factor β-catenin is released and translocates to the nucleus, where it activates several downstream transcripts [35] that prevent the formation of adherens junctions. For example, due to the decreased expression of claudin, the relocation of occludins triggers the disruption of tight junctions, desmosomes and gap junctions, which together allows individual cells to detach from the primary tumour [36]. This is followed by polarisation of the cytoskeleton and the cytoplasmic organelles [37].

Completed EMT results in 'front-to-back' cell polarisation, with spindle-shaped cells with the morphology of the mesenchymal phenotype, where the leading edges of invadopodia adhere to the ECM, with the secretion of proteases that enable cell invasion through the loosened matrix. Depending on the ECM stiffness, migratory cells undergoing the process known as mesenchymal-to-ameboid transition acquire an amoeboid, rounded shape morphology to squeeze through the ECM without disrupting it [38]. The heterogeneity of cancer cell populations allows for movements of either single cells (as in glioblastoma) or clusters of cells (as in carcinomas). The latter arises because not all of the cells complete EMT to the mesenchymal shape, and many adherens junctions remain in the EMT/MET hybrid states, which results in cell clusters lead by those cells that have completed EMT. Such cellular formations and collective movements have many advantages through all of the stages of metastatic processes [10]. Collective migration in most partial EMT cases is mediated by SLUG or SNAIL2, as observed in experimental animals and also seen in human cancers [39].

The ECM is an active component in mediation of cell-cell communication [40], and cell adhesion and/or movement, and since it has a highly dynamic structure, its components are constantly remodelled through hydrolases and proteases [41]. Proteases are essential to the migration mode of cancer cells, which actually respond to mechanical information about their ECM/tumour microenvironment and convert this information into chemical responses, which is known as mechanotransduction [42]. The basement membrane encircles benign tumours and represents an extreme of ECM stiffness, as it presents a barrier against the migration of EMT-induced epithelial cells. One way to circumvent this problem is to switch to the ameboid cell shape through a switch from MET to mesenchymal-to-ameboid transition [10,38,43]. The second option is that mesenchymal cancer cells proliferate long enough to exert mechanical stress along the membrane, to ultimately cause its rupture by what is known as anchor cell invasion [44]. Finally, EMT induces proteases, which include various matrix metalloproteinases (MMPs), ADAMs/ADAMTs and cathepsins [45], that are largely associated with invadopodia, and can act alone or in proteolytic cascades to facilitate cell invasion [46]. Elevated expression of various proteases, such as urokinase plasminogen activator (uPA) [47], MMPs [48] and cathepsins [49–51] has been linked to multiple cancers and correlated to poor patient prognosis [52]. Proteases have multiple target substrates, which enables cell invasion through interconnected cascades of proteolytic events, which is termed protease signalling [53,54]. For example, cathepsin B was shown to activate MMP-1 and MMP-3, which can then degrade their substrates, such as collagen and gelatin [55]. MMP-1 can activate proteinase-activated receptors, which are G-protein-coupled receptors that can drive cancer cell migration and invasiveness when activated [56]. Cathepsins B and L can also activate uPA, to cleave plasminogen into a broad-spectrum serine protease that can selectively degrade the ECM and activate MMP2, MMP3, MMP9 and MMP4, further propagating cell invasion. Elevated expression of various proteases, such as ADAMs and ADAMTs, and of a disintegrin and metalloproteases regulates cell adhesion, migration and fusion, with shedding of the ectodomains of membrane proteins, such as uPA. In addition, cathepsins, and especially cathepsins S and L, have been reported to act as shedases, as they can cleave off the extracellular domains of several receptors, like the EGFR [57–59].

Proteases that are induced by EMT can also be localised intracellularly; *i.e.* in the nucleus, cytosol and lysosomes. MMP2 activates the binding of octamer-binding TF 4 (OCT4) to its promotor, which leads to increased cell invasion and migration. ADAM17 and protease γ-secretase cleavage of the cell surface protein Trop2 and the Notch receptor in cancer and stem cells are most important for cancer progression, whereas other substrate modifications include ADAM17-promoted self-renewal, cell proliferation, survival and migration, and angiogenesis [60]. ADAM12 appears to be involved in EMT through the regulation of cell–cell adhesion in the epithelium, by cleaving claudin-3, -4, -7, occludins and E-cadherin, and by mediation of the release of endogenous EGF-ligands that induce EGFR signalling [61,62].

Lysosomal cathepsins prefer acidic environments, which are found in tumours due to intra-tumour hypoxia-induced glycolysis, also known as the Warburg effect [63]. This acidic milieu kickstarts their activity under otherwise suboptimal physiological conditions in terms of the extracellular space. Cathepsins B and X are associated with EMT-related appearance of a mesenchymal-like phenotype of epithelial breast adenocarcinoma. It was also demonstrated that expression of cathepsin B relies on TGF-β1, whereas cathepsin X expression appears to be independent of TGF-β1 during EMT [64]. As well as its association with EMT, cathepsin X can cause a switch in the migration mode of tumour cells, from mesenchymal to ameboid-like. Overexpression of cathepsin X in T lymphocytes promotes cytoskeletal rearrangements and morphological changes that are typical of MET, through the activation of β2 integrin receptor lymphocyte function-associated antigen (LFA)-1 [65]. It can be noted that cathepsin L can have a dual role (*i.e.*, suppression, oncogenesis), as its deficiency has been shown to promote tumour progression in mouse epidermis [66], whereas its nuclear activation in glioblastoma is essential to modulate the TFs that prevent cell apoptosis [67]. As proteolysis is an irreversible process, it has to be tightly regulated [45,54,68], ultimately by endogenous inhibitors [69,70], to preserve cell and tissue homeostasis [71]. However, cystatins might directly influence tumour progression *via* modulation of gene transcription, as has been described for cystatin E/M (reviewed by Breznik et al. [70]). This indicates that cystatins can interfere in signalling pathways, *e.g.*, cystatin C and TGF-β, to influence the MAPK/ERK signalling pathway [72] and the 14-3-3 protein pathway [73]. Another example here is stefin B, which has been shown to protect tumour cells against apoptosis and oxidative stress [74]. Moreover, cystatins can promote tumour progression by impairing antitumour immune responses, as was shown for cystatin F, which is an inhibitor of the major granzyme convertases cathepsins C and H in cytotoxic granules of effector immune cells. Cystatin F can be secreted from tumour cells or other cells into the tumour microenvironment, from where it can be internalised by cytotoxic cells, and can consequently inhibit their cytotoxic activities against tumour cells [70,75]. Altogether, this complex synergistic or anergistic signalling (Fig. 1) affects cancer cell EMT, whereby these cells acquire an intermediate epithelial–mesenchymal state.

### 2.2. The decisive role of the tumour microenvironment in EMT

Tumour heterogeneity has been recognised as one of the major obstacles to successful therapies, and it has both cancer-autonomous and cancer-nonautonomous origins. Cancer cell autonomous heterogeneity relates to genomic and epigenomic variations among cancer cells that accumulate during neoplastic cell evolution, to result in cancer stem cells [76] and to cause their plasticity [77]. Instead, cancer cell nonautonomous heterogeneity originates from the stromal component of the tumour, the tumour microenvironment. As has only been recognised over the past few years, this is closely related to the EMT-induced stemness characteristics of tumours, as reflected in the EMT–MET hybrid cell population(s) [10,78]. However, the EMT–MET

balanced phenotype is affected by microenvironmental factors, such as the ECM and hypoxia, and is moderated by communication between cancer cells and non-cancer cells within the tumour; *e.g.* endothelial cells, infiltrating immune cells and other cell types [79].

The tumour microenvironment contains different clones of cancer cells, as well as various types of stromal cells, which together modulate tumour progression and responses to therapies. The standard therapeutic approaches of irradiation and chemotherapy can eliminate the bulk of tumour cells and induce genetic alterations in the remaining dormant cells. For example, in glioblastoma, where the cells that survive acquire stem-like characteristics that are fostered by irradiation-induced changes in the tumour microenvironment, these are suspected to recur as glioblastoma stem cells (GSCs) [19,80]. Communication between cancer cells and stromal cells through paracrine signalling loops or direct cell–cell contact can affect therapeutic outcomes and lead to more aggressive tumour growth, as has been reviewed for glioblastomas [81].

The EMT-signalling pathways can be triggered in an autocrine manner, which generally occurs in oncogene/tumour suppressor-gene-transformed cancer cells, and in a paracrine/juxtracrine manner by the neighbouring cells, the 'stromal' cells, in the tumour microenvironment. The tumour microenvironment comprises local host-tissue stromal cells and infiltrating haematopoietic stem cells (HSCs), MSCs, and immune cells that secrete a wide variety of cytokines, chemokines and growth factors. Their mutual interactions were reviewed recently by Dongre and Weinberg [1], with several reviews in Cancers (2018), edited by Roche [9]. Superimposed on these, some systemic pathways (*i.e.*, hormonal and cellular infiltration from lymphatic and blood circulation) also have roles in the EMT–MET balance.

Not all stromal cells are associated with EMT of cancer cells. The suspects here are cancer-associated fibroblasts, CD4+ helper T cells, CD8+ cytotoxic T cells, regulatory T cells ($T_{reg}$ cells), myeloid-derived suppressor cells and tumour-associated macrophages. Some examples were briefly summarized by Jing et al. [82], who indicated that cancer-associated fibroblasts can influence the onset of EMT epigenetically through DNA methylation, to favour the expression of EMT-linked genes in cancer cells. Tumour-associated macrophages produce TGF-β, which can trigger EMT either alone or in addition to tumour necrosis factor (TNF). Tumour-associated macrophages also release interleukin (IL)-6, which leads to activation of cyclooxygenase-2/prostaglandin $E_2$ (COX2/PGE2) and β-catenin signalling, which both promote EMT. The SNAIL TFs are known to promote inflammation by up-regulation of pro-inflammatory signals, such as IL-1, IL-6, IL-8 and IL-10, which maintain the inflammatory state in the cancer milieu. In response to tumour-associated macrophage secretion of EGF, tumour cells induce paracrine cross-talk that affects them in turn, and that causes tumour-associated macrophage transition to the active M2 state, which co-operates in metastasis formation. $CD4^+$ and $CD8^+$ lymphocytes repress E-cadherin in neighbouring cells while up-regulating ZEB1 and vimentin. Myeloid-derived suppressor cells accumulate at the front of a forming tumour, where they produce TGF-β, HGF and EGF, and activate COX2, thus triggering EMT in the surrounding cells, which can lead to metastasis formation [82].

Taken together, this complex intercellular cross-talk can create various types of gradients, from oxygen, nutrients and chemokines, to proteases and their antagonists. These gradients are balanced by the biophysical constraints of the ECM, whereby some, such as the basement membrane, are loosened, thus allowing metastatic spread *via* the systemic and local vasculature. Here, hypoxia and altered glucose metabolism due to the Warburg effect have very important roles [63]. Hypoxia can induce the TF HIF-1α that promotes EMT through the induction of TWIST, SNAIL1 and ZEBs expression, which then leads to down-regulation of E-cadherin. Altogether, this complex synergistic and anergistic signalling (Fig. 1) promotes cancer cell EMT, where they then acquire an intermediate epithelial–mesenchymal state.

## 3. Cancer stem cell plasticity and metastasis formation

An interesting concept is that EMT generates cancer cells, which appear to reside in an intermediate state along the epithelial–mesenchymal spectrum, as hybrid epithelial and/or mesenchymal phenotypes. It is unclear how many distinct combinations of stroma-derived cues are needed to stabilise these carcinoma cell states. Understanding the signalling events that are required to create and maintain this dynamic equilibrium among EMT-induced quasi-mesenchymal states is still scarce. However, such 'cell plasticity' has also been described for CSCs, where they can express the stemness markers and undergo signalling similar to that described above for EMT. CSCs are a subpopulation of malignant cells within a tumour that have the ability to self-renew through symmetric cell division, and to differentiate into diverse cell types by asymmetric cell division. In a heterogenous tumour bulk, CSCs are the only subpopulation of cells that has tumourigenic potential, and also drug and irradiation resistance [45,76,80,83,84]. CSCs usually share many features with normal stem cells, such as their relative quiescence when located in their specific microenvironment, known as their 'niche'. While normal stem cells have low genetic stability, that of CSCs is high, and they show greater resistance to several therapeutic regimens compared to non-CSCs from the same tumour.

Increasing experimental observations have suggested that EMT is linked to stem-cell properties, with EMT identified as a critical regulator of CSCs [16,78,85,86]. Where CSCs appear and stabilise across the epithelial *versus* mesenchymal phenotype spectrum probably depends on the EMT-TF combinations involved in each type of cancer [1]. CSCs thus express a combination of epithelial and mesenchymal markers and traits, as they undergo only partial EMT and retain an intermediate state along the epithelial–mesenchymal spectrum. The current view is that stemness features are not simply associated with a more epithelial or more mesenchymal phenotype, but are intermediates in the so-called metastable EMT states [34], although with a tolerance to some cell plasticity [11]. As a consequence, CSCs can persist after anticancer therapies and can serve as the founders of the metastatic colonies that can lead to tumour relapse [78]. The coupling between EMT and stemness is finely regulated. Jia et al. [87] formulated a mathematical model to analyse the dynamics of the coupled decision-making circuits of EMT-ZEB/miR-200 and stemness–LIN28/let-7. This model suggested that the 'stemness window' most likely lies at an intermediate position along the EMT axis with the epithelial and mesenchymal phenotypes as the two ends, whereas the hybrid epithelial–mesenchymal phenotypes appear to be resilient to therapies such as chemotherapy, and to possess plasticity, all of which are closely related to stemness traits.

Experimental activation of EMT has been seen for overexpression of TWIST1 and SNAIL, or for treatments with TGF-β, which confers many of the properties of CSCs [85,88]. These include CSC-specific stemness marker expression, which as well as including the commonly recognised OCT4/2, SOX2, CD133, Notch, Musashi and Nestin, might also include the elevated CD44 and reduced CD24 glycoproteins, depending on the type of cancer. However, *in vitro*, all CSCs share greater ability to form spheres and organoids and to seed tumours of the same histology in mice, which represents *sine qua non* proof of their stemness. In addition, CSCs in EMT-like processes transform into highly migratory metastatic CSCs (mCSCs) that can establish new micrometastases at secondary organs [77,89]. Reciprocally, non-CSC cancer cells in tumours have been shown to de-differentiate into CSCs [90], which highlights the plasticity and bidirectional interconversion between these two populations. This appears to be due to EMT-like processes, as CSCs in breast cancer, for example [88], have characteristics associated with cells that have undergone EMT [16].

### 3.1. EMT in metastasis formation

Visvader et al. [91] proposed that metastatic CSCs might exist and have properties distinct from primary CSCs. This concept that metastatic colonies at secondary sites originate from CSCs [1] indicates that the EMT-like programme is not only needed for the initial steps in metastatic processes, but also to enable metastatic cells with CSC characteristics to adhere and home in on secondary sites. As the EMT programme imparts heritable phenotypic changes to carcinoma cells through epigenetic modifications without introducing new genetic alterations, the reverse process, MET, at the secondary site should restore the original CSC traits, to allow them to regrow the original tumours. However, these CSCs will be in a different microenvironment in terms of the metastatic niche in the secondary organ, which might prevent them from successful colonisation of another organ. In the secondary organ, the process might be halted in an intermediate EMT/MET state, which will result in slowly proliferating or dormant cells, arrested in G0/G1 phase, as indeed shown for micrometastases in metastatic niches [92]. These CSCs might then remain for a long time, until their proliferation is activated to colonise the secondary organ [93]. Inherent genetic and genomic instabilities in metastatic cells (*i.e.*, CSCs) might lead to the evolution of cells that would finally be able to gain colonisation potential in any of the preferential secondary tissues [94]. EMT-like processes in dormant CSCs induced by activating signals from the new metastatic microenvironment will initiate mCSC clones that can colonise the secondary organ [15]. A large body of evidence indicates that dormant metastatic cells have the characteristic plasticity of CSCs (reviewed in [14,95]). The plasticity of resident metastatic cells due to the acquisition of the partial EMT/MET phenotype is thus an emerging concept [86,96]. However, the reversibility of these processes allows for co-existence of both types, as CSCs and mCSC, in metastatic niches [97]. Altogether, by manipulating the dormancy-regulating processes it might be possible to suppress the colonisation of disseminated metastatic tumour cells.

Research on the targeting of dormant metastatic cells is not only focused on dormant CSCs, but also on identification of the tumour tissue niches that promote CSC dormancy. However, the following unresolved question remains: does one mCSC type seek a niche that is similar to the primary tumour or a *de-novo* niche, to accommodate micrometastases. The metastatic stem cell niche microenvironment has a detrimental role in metastatic colonisation. Recently, Prager et al. [77] suggested a flexible model where CSCs pro-actively remodel their microenvironment to maintain a supportive niche where they can sustain their stemness characteristics. Examples of mutually supportive CSC/niche interactions have been seen in the hypoxic niche, the immune niche, the perivascular niche and the CSC-infiltrating region [77]. However, these niche properties might also overlap, as shown on Fig. 2. In the hypoxic niche [98], cancer cells induce signalling of the TF HIF and up-regulation of stemness markers, such as CD44 and Notch signalling. Further, several studies have proposed that hypoxia promotes a quiescence phenotype in CSCs, to facilitate resistance to therapies [99]. The perivascular niche is characterized by interactions with endothelial cells and components of the ECM, whereas CSCs, in turn, are drivers of vascularization *via* both stimulation of endogenous endothelial cells and vascular mimicry [100] and by transdifferentiation into pericytes. The stroma of these niches is composed of HSCs and MSCs and various types of their progenitors, and immune cells and fibroblasts in perpetual communication, mediated by cytokines, exosomes and gap junctions. Of note, metastatic niches in these distant organs can even evolve from a pre-metastatic niche if soluble messages are received from the primary metastatic cells [101]. In the light of the classic 'seed and soil' theory of cancer dissemination, the 'right soil' represents the metastatic stem cell niche [102]. These niches are not mutually exclusive, as has been described in detail for glioblastoma (see Section 3.2.).

### 3.2. The glioblastoma stem cell niche microenvironment

Glioblastoma (GB) is the most aggressive and therapeutically non-responsive primary brain tumour in human [103]. GBs are of neuroectodermal origin [104], and appear to arise from astrocytes, which develop into secondary GB (WHO grade IV stage), whereas primary, or *de-novo*, GB appears without any earlier premalignant stage. Primary GB can arise through transdifferentiation of normal neural stem cells, or even by de-differentiation from neurons [105]. The standard-of-care treatments for GB include surgical resection, radiotherapy, chemotherapy and biological therapeutics, although these can at present only slightly enhance patient survival, presumably due to the therapeutic resistance of GSCs, and also to high GB heterogeneity and highly invasive intracranial and even metastatic GB spread [15]. Moreover, GB frequently shifts its biological features upon recurrence, to become more aggressive and invasive [80,106], as the phenotype that is associated with mesenchymal GB features [107]. Although of non-epithelial origin, EMT-like transition and mechanisms have been observed in GB and are associated with increased characteristic diffuse GB cell infiltration into the brain parenchyma, as well as with extreme resistance to conventional treatments [80,108,109]. The TFs involved in EMT-like processes in GB are similar to those in carcinomas; *i.e.*, SNAIL1 and SNAIL2, ZEB1 and ZEB2 and TWIST [110,111]. Gene silencing of *SNAIL* reduces GB cell invasion, migration and proliferation [112], whereas *ZEB1* and *ZEB2* are correlated with the invasive phenotype, tumour grade, therapeutic resistance and poor survival of patients with GB [111]. In the central nervous system, the basement membrane only comprises the vascular walls [109], and the expression of E-cadherin in GB cells is relatively low, although it appears again in GSCs, and more so in aggressive mesenchymal GB cells [108]. In contrast, N-cadherin is highly expressed in astrocytes, where it regulates their polarity and migration, but it enhances GB cell migration [113]. The master EMT-like-signalling pathways in GB are the TGF-β and Wnt/β-catenin pathways and signalling by specific TKRs [114], such as the EGFR, fibroblast growth factor receptor and platelet-derived growth factor receptor [23,115]. This increased expression of known mesenchymal markers includes vimentin, fibronectin, CD44 and collagen, as well as activated kinase receptor signalling [19,116]. TGF-β drives GB EMT activation through SMAD-dependent or SMAD-independent pathways [116]. Joseph and co-workers [19] showed that TGF-β induces a mesenchymal shift in GB cells through the concomitant increased expression of ZEB2, which results in morphological changes to GB cells due to increased collagen COL5A1 and fibronectin; TGF-β signalling *via* SMAD2 increases GB cell invasion. The stemness-related canonical Wnt/β-catenin pathway is also activated in an EMT-like process in aggressive gliomas [117] and in the cells at the invasive edges of GB tumours, compared to the central GB regions [118]. Multiple Wnt/β-catenin targets are then overexpressed in mesenchymal GB subpopulations, such as CD44, the TF Runx2, WNT-ligand receptor Frizzled-1 (FZD1) and Dickkopf-1 (DKK1) [118,119]. Hypoxia mediated through the HIF-1α–ZEB1 axis and the HGF/c-MET signalling pathway also promotes the mesenchymal shift and GB cell invasion [19]. This is of particular relevance for niche-associated GSCs (see below), which can activate several intracellular downstream signalling pathways to further promote EMT, such as the PI3K/AKT, RAS/MAPK and Wnt/β-catenin pathways [12]. Similar to Wnt ligands, c-MET is also expressed in GSC populations and maintains the GSC phenotype, which is most relevant for GB/GSC radio-resistance [120]. Also, HGF7/c-MET signalling can induce the invasive properties of GSCs through direct activation of Wnt/β-catenin signalling [12]. Taken together, the EMT-like processes in GB result in the invasive, mesenchymal GB/GSC phenotype, although the signalling is less clear than in epithelial carcinomas.

Glioblastoma stem cells were among the first discovered and isolated CSCs [121] and have also been one of the most investigated. They contribute to GB tumour initiation [77,83,84,105,122] and therapeutic

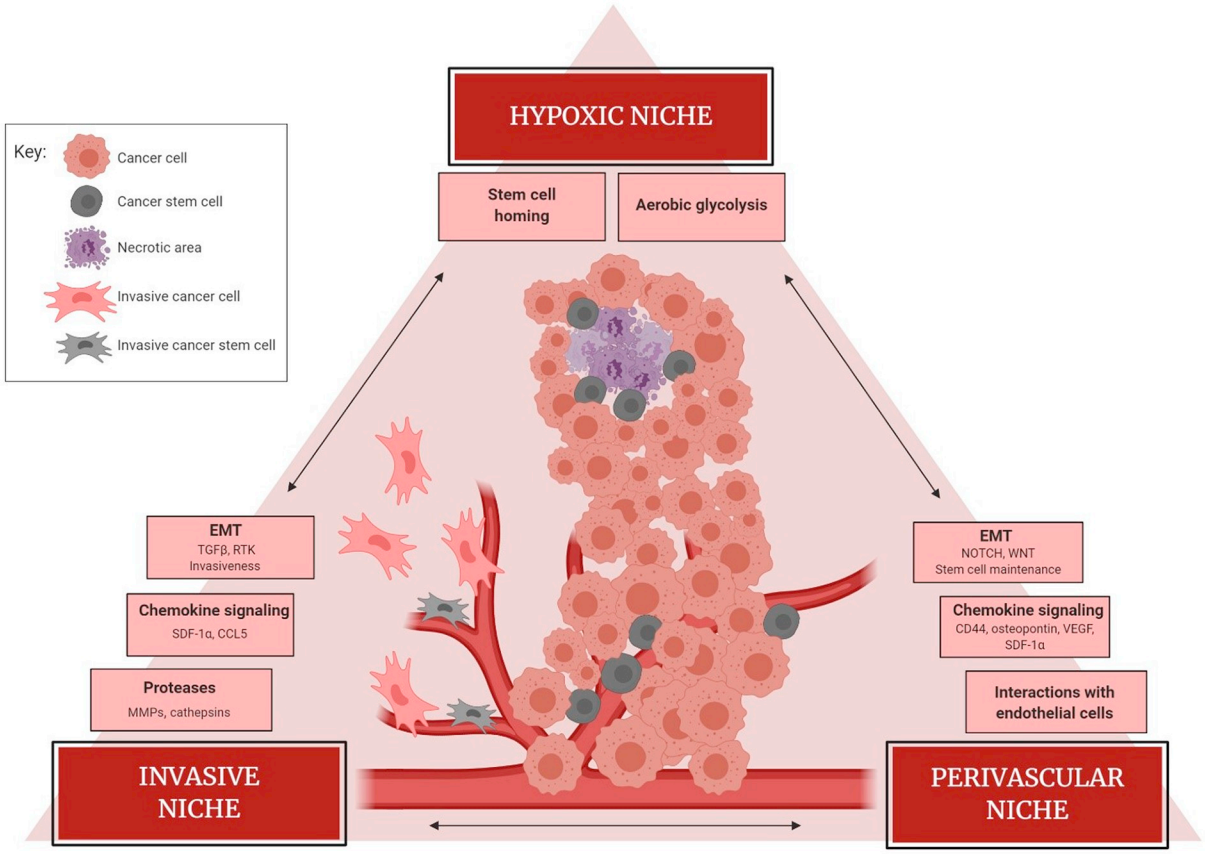


**Fig. 2.** Different types of glioblastoma stem cell niches.
Characteristics of the three types of glioblastoma stem cells (GSCs) described in the literature, and the GSC niches, including hypoxic, perivascular and invasive niches. Hypoxic regions in the fast-growing tissue directly activate aerobic glycolysis, lowering the pH, and in coordination with oncogenes and tumour-suppressor genes, this affects the regulation of GSCs through hypoxia inducible transcription factors (HIFs). In the perivascular niche, GSCs interact with endothelial and other stromal cells that are responsible for stem cell maintenance. These can promote angiogenesis *via* vascular endothelial growth factor (VEGF) secretion. These three types of niches are to a certain extent overlapping, as hypoxic areas can be located adjacent to perivascular niches, and they share the chemokine-receptors axis. The chemokine SDF-1α is important for recruitment and retention of GSCs in the niche, which acts through CXCR4 receptors; on the other hand, osteopontin-CD44 signalling enhances the stem cell phenotype. The invasive niche is characterized by invasive cancer cells that migrate deep into the brain parenchyma, where proteases, chemokine signalling and EMT have important roles.

resistance, due to their active DNA damage response mechanisms [123] and high expression of the ATP-binding cassette (ABC) transporters [124]. Furthermore, GSCs can evade the immune response [125,126]. GSCs are identified by markers, the main ones of which are CD133, CD15, SOX2, NANOG, OLIG2 and Nestin [121,127,128]. We have reported on a new selective GSC marker, tetraspanin CD9, that discriminates between GSCs and normal neural stem cells [129].

GSCs are mainly, although not exclusively, localised in specific niches that protect their dedifferentiated state and presumably protect them from therapeutic insults and immune cells [93,124,130]. Paracrine interactions with cancer-associated fibroblasts increases the cytokine-activated Wnt and Notch signalling pathways, which are both implicated in stem cell maintenance [93]. MSCs increase GSC proliferation [131], and maintain GSCs through the IL-6/gp130/STAT3 pathway [132]. Another reported pathway in GSCs is NF-kB, which is promoted by stromal MSC-derived SDF-1α, IL-6 and IL-8 [133].

Morphologically and functionally, GSC niches are particularly distinct (Fig. 2). We have recently demonstrated similarities between GSC and HSC niches in bone marrow, both of which contain MSCs and share the functional chemo-attractive proteins and their receptors [134,135]. We have shown that GSC niches are both peri-arteriolar and hypoxic, where $CD133^+$ GSCs are localised adjacent to the *tunica adventitia* of a small subset of arterioles. These create hypoxic areas, as arterioles do not take part in oxygenation. So these hypoxic conditions can nourish the stemness of GSCs. Therefore, the hypoxic peri-arteriolar GSC niche is a logical explanation for this seemingly contradictory need for both hypoxic conditions and the presence of mature endothelial cells. MSCs, smooth muscle cells and other stromal cells release chemokines such as stromal derived factor-1α (SDF-1α), osteopontin and CCL5, whereas GSCs express their receptors. Breaking this cytokine–chemokine axis by activation of abundant cysteine cathepsins B, K and X in the niches [136] might represent a strategy to release and activate dormant GSCs from the niche, which would then differentiate into rapidly dividing progenitors that are more vulnerable to radiation [137] (Fig. 3). Ongoing clinical trials aim to block the CXCR4 receptor to mobilize leukaemia stem cells out of the HSC niches, to sensitize them to chemotherapy [138] (ClinicalTrials.gov: NCT00512252). Similar treatment approaches need to be investigated clinically to improve GB therapies.

## 4. Clinical applications: Diagnosis and reprogramming EMT in cancer-cell targeting

The transient and dynamic nature of EMT complicates its status as a tool for diagnosis and/or prognosis [139]. For diagnosis, it is crucial to identify genetic or protein EMT signatures that can be used to distinguish between the epithelial and mesenchymal phenotypes of cancer cells. For example, E-cadherin down-regulation is a diagnostic EMT hallmark, along with overexpression of N-cadherin, vimentin and other mesenchymal markers, which can thus provide insights into cancer progression [140]. Immunohistochemical labelling for E-cadherin

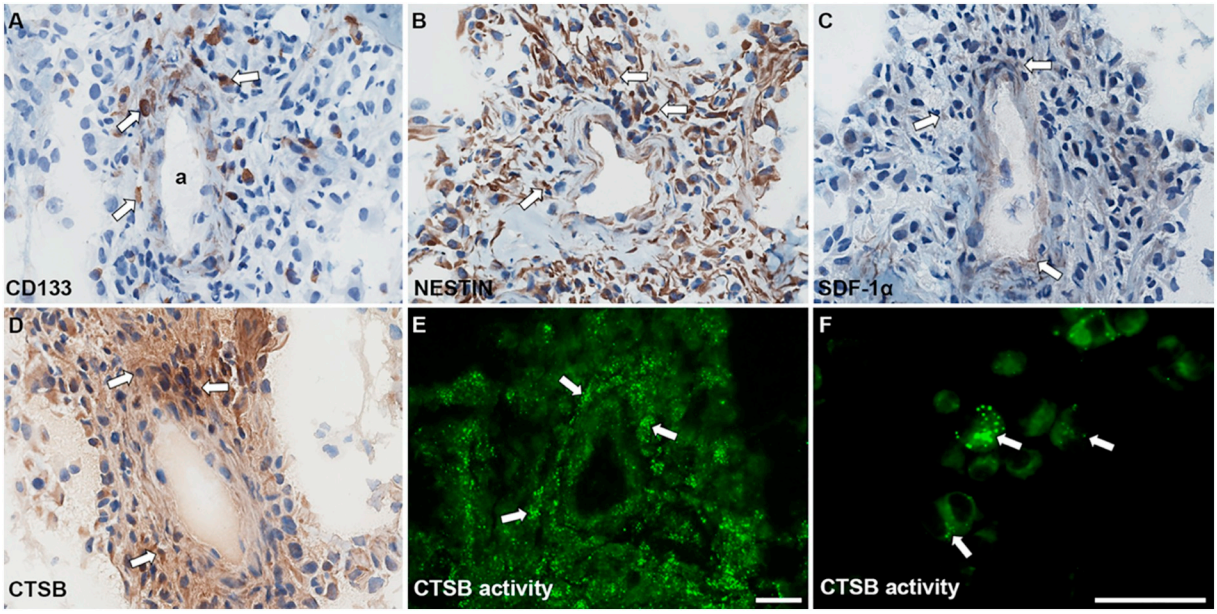


**Fig. 3.** Cathepsin B is expressed and active in glioblastoma stem cell niches.
Immunohistochemical staining for glioblastoma stem cells (GSCs) have been reported in the literature, and it has been revealed that GSCs are more abundant in certain locations, known as niches, that are different from the surrounding tumour microenvironment, as shown on Fig. 2. GSCs were detected in peri-arteriolar regions of glioblastoma tissues, where they were identified by stem cell markers, such as CD133 (A) and Nestin (B) in these images. The chemotactic cytokine SDF-1α is also present in these regions (C; white arrows). Cathepsin B protein (D) and activity were localised in peri-arteriolar GSC niche regions (E) and in GSCs *in vitro* (F; white arrows). Immunohistochemical labelling of proteins was performed with DAB as the chromogen (brown colour). Cathepsin B activity was detected as green fluorescent dots using metabolic mapping and a selective cathepsin B substrate [170]. *a*: arteriole. Scale bar: 50 μm.

shows loss of expression in almost all lobular carcinomas tested *in situ*, although not in ductal breast carcinoma [141]. N-cadherin over-expression was also found in patients with lymph node metastasis in gastric tumours, who had poor prognosis [142]. Other molecular players in carcinomas that can be used for EMT validation are: β-catenin relocation to the cytoplasm or nucleus during progression of colorectal cancer [143]; and p120 catenin in the adherens junction is re-localised in breast tumours and other carcinosarcomas [144], including close to 500 human tumours collected from patients. In basal-like breast carcinosarcomas, the EMT markers vimentin, N-cadherin and cadherin-11 (which mediates $Ca^{2+}$-dependent cell–cell adhesion) are up-regulated, whereas E-cadherin and several cytokeratins that are used for subtyping of carcinoma progression were reduced [144].

Visualization of EMT is another approach that might serve diagnostic or research purposes. Expression of mesenchymal-state-related proteins thereby offers an opportunity for EMT visualization by selective staining. Single-domain antibody fragments (*i.e.*, VHH antibodies) or nanobodies represent more effective alternatives to conventional antibodies due to their structural simplicity. These can be easily expressed in transfected cells and have an advantage over fluorescent fusion proteins, which can create serious artefacts [145,146]. Vimentin is an intermediate filament protein that is expressed in mesenchymal cells and also during metastatic progression in cancer cells undergoing EMT. It is concentrated at protrusions at the leading edge of migrating cells, and it has been successfully visualized in mammalian cells undergoing EMT after TGF-β induction. Specific nanobodies have been expressed and fused with green fluorescent protein (known as chromobodies) to visualize vimentin without any specific aggregation or changes in morphology [145]. Another nanoprobe successfully predicted the therapeutic effects of curcumin on tumour xenograft models in mice [147].

### 4.1. Therapeutic resistance

The above-described observations strongly suggest that the EMT programme and the CSC phenotype are closely associated, although they can also be uncoupled from one another under certain conditions *in vivo*. More investigations of the association and distinction between EMT and the CSC state are thus required to fully exploit the EMT–CSC link for therapeutic purposes. There appears to be great potential in targeting EMT as part of these therapeutic strategies, to reduce migration of cancer cells and metastasis formation. Even if this might not be completely effective on its own, efforts towards targeting EMT might help to at least a certain degree to re-sensitize resistant tumours to treatments. Additionally, targeting EMT with visualization probes would contribute to better understanding of this cellular programme, and help in predicting drug efficacies at the cellular level.

Epithelial-to-mesenchymal transition has been linked with increased therapeutic resistance. EMT confers chemoresistance, radioresistance and resistance to immunotherapy. For chemoresistance, EMT is often associated with multidrug resistance phenotypes [148]. The first hint that EMT might be connected to drug resistance was obtained when specific antibodies against TGF-β (an EMT inducer) restored the drug sensitivity to alkylating compounds in mouse mammary carcinomas [149]. Cells undergoing EMT overexpressed the ATP-binding cassette (ABC) transporters, which can increase drug efflux and subsequently lower intracellular drug concentrations [148,150]. The link between overexpression of ABC transporters and EMT is further supported by the binding sites on ABC transporter promotors for EMT-TFs, including TWIST and SNAIL [151]. SNAIL1 and SNAIL2 are involved in the acquisition of resistance to radiotherapy and paclitaxel in ovarian cancer cells. They also both participate in p53-mediated apoptosis through active repression of pro-apoptotic genes and indirect activation of the self-renewal programme through de-repression of promotors for self-renewal genes, which provides resistance to cellular stress caused by radiotherapy and chemotherapy [152].

Human and murine melanoma cells that were initially transduced with SNAIL showed typical EMT features and increased immunosuppression by induction of regulatory T-cells, which enforce negative regulation of other immune cells, and impair antigen-

presenting dendritic cells. These effects were reported *in vitro* and *in vivo* in mouse models. SNAIL$^+$ melanomas are resistant to dendritic-cell-based immunotherapies, whereas the same therapies are effective on SNAIL$^-$ melanomas, which confirms the involvement of EMT in immunotherapy resistance. Moreover, when SNAIL-specific small-interfering RNAs were injected into tumours, they inhibited tumour growth and metastasis, and a renewed anti-tumour immune response was also observed, due to the increased infiltration of cytotoxic T lymphocytes, which overcame the tumour cell immunosuppression [153].

### 4.2. Anti-EMT therapies

Despite difficulties when it comes to targeting EMT with cancer therapies, there have been a few studies that have focused on investigations into treatments *via* the targeting of EMT. An integrin-linked kinase (ILK) involved in activation of the AKT pathway, which leads to EMT, was targeted with emodin (1,3,8-trihydroxy-6-methylantraquinone), which resulted in MET in ovarian cancer cells [154] and in reduced EMT in breast cancer cells, as they could not undergo downstream phosphorylation through the ILK/Gsk3/Snail2 and ILK/Akt/mTOR signalling pathways, respectively [155,156].

Cyclopamine is a steroidal alkaloid from the corn lily *Veratrum californicum* that can inhibit Hedgehog signalling in cells undergoing EMT. Aberrant Hedgehog signalling up-regulates SNAIL through Notch signalling and TGF-β1, which then induces EMT and enhances cancer progression. The modified KAAD-cyclopamine (3-keto-N-(aminoethylaminocaproyl-dihydrocinnamoyl) cyclopamine) then showed 10-20-fold higher potency than the natural compound [157].

Metformin is already being used for reducing glucose levels in type II diabetes, and it has been shown to inhibit TGF-β–induced EMT, which was confirmed by prevention of E-cadherin down-regulation, and inhibition of increases in N-cadherin and vimentin. The mechanism here appears to involve miR30a, as its levels were up-regulated and it was also shown that miR30a targets SOX4, which is associated with EMT initiation [158,159]. Moreover, metformin was shown to decrease the dose of chemotherapy with doxorubicin to prolong tumour remission in mouse xenografts. It also prevented relapse when combined with paclitaxel and carboplatin [160].

Another promising approach is the use of RNA interference with miRNAs and agomiRs/antagomiRs. In prostate cancer cells, miR-875-5p was reconstituted, while it is down-regulated in cancer cells and its levels correlate with those of E-cadherin. Through EGFR targeting, which has an established role in maintenance of EMT and DNA repair after radiotherapy, reconstitution of miR-875-5p led to re-sensitisation of prostate cancer cell lines and xenografts to radiotherapy. EGFR inhibition was probably the main culprit for ZEB1 down-regulation, which impairs homologous recombination-dependent DNA repair [161]. In another study, inhibition of vascular endothelial growth factor (VEGF) receptor expression by an artificial miRNA resulted in reduced cell proliferation, increased apoptosis, and reduced cell migration and invasion in pancreatic cancer cell lines and a mouse xenograft model. In the mouse model, VEGF receptor silencing had synergistic effects with cisplatin chemotherapy [162]. In malignant melanoma cells, reinforced vaccine efficacy of B16F10/GPI-IL-21 was noted when administered with the short hairpin ZEB1 RNA (shZEB1) or miR-200c agomiR. Treatment with miR-200c agomiR resulted in ZEB1 silencing. Also, concurrent inhibition of EMT by RNA interference and application of a vaccine resulted in elicited anti-tumour immunity in B16F10-melanoma-bearing mice [163].

Drugs that target epigenetic regulation of EMT represent a potentially powerful approach that can be used alone or in combination with conventional therapies [164]. The pan-deacetylase inhibitor panobinostat might influence the differentiation status of human hepatocellular carcinoma cells *in vitro* and *in vivo* in a xenograft model. It increased the differentiation/epithelial markers and decreased the levels of the dedifferentiation/mesenchymal markers vimentin and sonic hedgehog homologue/patched (SHH/Ptc), parallel with prognostically favourable expression of β-catenin [165]. Sorafenib is already being used in clinical treatments [166], and it not only interferes with EMT *via* direct inhibition of targeted kinases, but it can also reverse changes in histone modifications that occur during EMT. Sorafenib caused the loss of active histone markers at promotors for TGF-β1, SMAD2/3, SNAIL1 and SNAIL2 in human lung epithelial cells undergoing TGF-β1–induced EMT [169]. Mocetinostat is a histone deacetylase inhibitor that in pancreatic adenocarcinoma cells can reverse drug resistance and repress stemness properties through lowering ZEB1 expression and increasing miR-203 expression, which is inhibited by ZEB1 during EMT [167]. Selective inhibitors of lysosomal cysteine proteases, such as inhibitors of cathepsins B and X, have been shown to inhibit EMT-related tumour-cell migration and invasion [168,169]. Several selective cathepsin B inhibitors have already been tested in different preclinical tumour models, and been shown to inhibit cell migration, ECM degradation and invasion of tumour cells *in vitro*, as well as tumour growth in mice [168].

## 5. Conclusions

Epithelial-to-mesenchymal transition is an essential molecular and cellular process in normal embryogenesis and wound healing, although it also has adverse consequences for the outcomes of cancers. In malignant progression, many different signalling pathways activate and regulate EMT, where the mainly irreversible oncogenic transformations are superimposed on reversible epigenetic transitions. These latter can affect transcription- and translation-controlling factors, as has been shown mainly in carcinomas. EMT appears to proceed in several molecular and cellular steps, to gradually change the phenotype, although it appears that in all of the steps, it is opposed by the potential reverse MET process. In cancers, the EMT–MET balance generally does not go to completion. This results in a hybrid epithelial/mesenchymal phenotype that contributes to the so called 'epigenetic heterogeneity' observed among carcinoma cells. The major outcome of EMT is related to increased cancer cell invasiveness, and is associated with homotypic cell-cell detachment, ECM degradation by induced proteases, and cell migration. Another phenotypic change that have so far only been seen upon EMT in cancer cells is a gain in stemness characteristics. The metastable hybrid EMT–MET phenotypes express high levels of stemness markers, which are known to also appear in CSCs. These provide CSC characteristics, such as therapy resistance and asymmetric divisions, as well as variable degrees of cell plasticity. The EMT–MET balance also has a crucial role in the stop-and-go of metastatic cell populations that evolve from primary dormant CSCs.

The tumour microenvironment has a decisive impact on both protection of CSCs/mCSCs homing in on their niches and on the selection of metastatic cell subpopulations, even in the last step of colonisation of a secondary organ. The coexistence of diverse microenvironments throughout solid tumour progression generates and selects for heterogeneity within the CSC population, represented by clusters of metastable EMT–MET hybrids. Furthermore, CSCs sustain the niche environment and represent the pool from which metastatic cells are selected to metastasise in a secondary organ. There again the plasticity of the hybrid EMT–MET phenotypes allows them to invade and settle as micrometastases, although in a new metastatic niche microenvironment. Finally, a role for EMT in organ colonisation, *i.e.*, regrowth into a secondary tumour, is suspected, although not fully understood yet.

In this review, we also detailed the above-described phenomena for progression of the non-epithelial cancer, glioblastoma, with emphasis on the importance of EMT in GSC biology. GSC niches are among the most investigated, where we recently suggested close similarity to the haematopoietic niche, and defined the cellular mechanisms of homing into the hypoxic/perivascular niches.

These concepts have clinical applications for EMT-related diagnostic

markers, their imaging, and targeting for cancer treatments. New pharmacological approaches in multi-targeted cancer therapies through the reprogramming of EMT–MET were also discussed.

## CRediT authorship contribution statement

**Bernarda Majc**:Writing - review & editing.**Tilen Sever**:Writing - review & editing.**Miki Zarić**:Writing - review & editing.**Barbara Breznik**:Writing - review & editing.**Boris Turk**:Writing - review & editing.**Tamara T. Lah**:Writing - review & editing.

## Declaration of competing interest

All authors declare that they have no financial interest and no personal relationship considered potential competing interest.

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