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Novel Therapeutic Strategies: Targeting Epithelial-Mesenchymal Transition in Colorectal Cancer

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Novel Therapeutic Strategies: Targeting Epithelial-Mesenchymal Transition in Colorectal Cancer

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Abstract

Epithelial-mesenchymal transition (EMT) is a process during which cells lose their epithelial characteristics, for instance apical-basal cell polarity and cell–cell contact, and gain mesenchymal properties, such as increased motility. In colorectal cancer (CRC), EMT plays an important role in tumour progression, metastasis, and drug resistance. ~~In this review, we describe the accumulating evidence from preclinical and early clinical studies which show that EMT markers may serve as outcome predictors and potential therapeutic targets in CRC.~~ There have been accumulating evidence from preclinical and early clinical studies which show that EMT markers may serve as outcome predictors and potential therapeutic targets in CRC. This review decribes the fundamentals of EMT which include the biology of EMT and the newly-minted partial EMT (pEMT), and their associated changes. We have also provided a comprehensive summary of therapeutic compounds capable of targetting EMT markers, ranging from preclinical stage, clinical trials or repurpose potential. Lastly, we have also explored on the potential obstacles that lie ahead of EMT bench to bedside drug development.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignant tumour worldwide, accounting for more than 9% of annual cancer mortality[1]. Although advances have been made in screening, early detection and management of established disease, targeted therapeutic innovations have been relatively sparse. It is therefore widely acknowledged that a deeper understanding of the complex biology of CRC must precede rational drug development.

Epithelial-mesenchymal transition (EMT) was first described in embryogenesis and refers to a cellular reprogramming process in which epithelial cells acquire a mesenchymal phenotype. EMT plays an important role in development, wound healing, and malignant progression in which cancer cells are endowed with properties associated with more aggressive phenotypes[2].

Upon activation of EMT, tumour cells undergo a series of physical changes including tight

junction dissolution, disruption of apical–basal polarity, and reorganization of the cytoskeletal architecture, all of which facilitate the dissemination of cells from their primary site, invasion of surrounding tissues, survival in the general circulation, leading finally to the formation of metastases in distant organs. In addition, recent studies have attributed increased resistance to chemotherapy and immunotherapy to EMT as it promotes interaction with tumour-associated stromal cells, known for their protumorogenic properties[3,4]. ~~(Figure 1).~~

EMT-associated changes

EMT is a generic description integrating several processes which share certain common features, but which can vary according to the tissue origin, stromal cell components and environmental cytokines. EMT is multifaceted, often reversible and involves a range of changes in cell biology, gene expression and physiology. ~~Notably~~Traditionally, EMT is though to be a binary process. However, accumulating evidence has shown otherwise.~~,~~ EMT programming runs on a spectrum;~~-~~ it can range from complete EMT/MET to cells with incomplete suppression of pre-existing epithelial characteristics and incomplete acquisition of mesenchymal ones, a hybrid intermediate stage known as partial EMT (pEMT)[5]. In the intervening years, heterogenous populations with different degree of EMT can be observed within tumours and pEMT has been widely observed across a broad range of cancers. Recent evidence has proven that cancer populations with pEMT have the highest metastatic potential. Interestingly, among these hybrid subpopulations, those with more epithelial features and less mesenchymal conversion have the greatest malignancy and metastatic potential[6,7](Figure 1). It has been reported that patients with primary prostate, breast or lung cancers that exhibit the full range of epithelial-mesenchymal plasticity, that is, transcriptional evidence of both EMT and MET, have the poorest outcomes[8].

Cellular changes Increased motility and migration through extracellular matrix (ECM) is a typical feature of cells undergoing EMT, during which epithelial cell-cell junctions are dissolved, and the integrity of their basement membranes compromised. These changes are accompanied by alterations in cell polarity, cytoskeletal rearrangements, and a switch in expression from mainly keratin to mainly vimentin. Morphologically, this leads to a striking loss of the typical polygonal, cobblestone appearance of epithelia and the emergence of spindle-shaped fibrous cells which express mesenchymal cell markers, eg. neural cadherin (N-cadherin), vimentin and fibronectin and cell-associated matrix metalloproteinase (MMP) activity[9].

Genetic changes A number of highly conserved transcription factors (EMT-TFs) have been identified, such as Snail, Slug (Snail2), Zeb1, Zeb2, and Twist, which are major regulators of EMT programs, functioning in a variety of cell-specific combinations with each another. In addition, a number of microRNAs (miRNAs) as well as non-coding RNAs can regulate, and be regulated by, the key EMT genes and influence EMT programming. Among these non-coding RNAs, miR-200 family and miR-34 family are the most researched (Figure 1). For example, miR-200c targets *Zeb1* to suppress its expression,

thus inhibiting migration and invasion of CRC[10]. These interrelationships leads to numerous permutations which call for the need to resolve them for better understanding of CRC EMT.

Physiological changes Many studies have demonstrated an association between EMT and acquisition of stem cell-like properties, characterised by expression of classical surface markers ~~(CD44^{high}CD24^{low})~~. CD51, a putative cancer stem cell marker in CRC, has been found to be one of the most frequently expressed markers[11]. CD44- and SOX2-enriched tumorspheres have been detected in TGFβ1-treated CRC cells[12]. These~~is~~ acquisitions confer~~s~~ a range of properties including relative anticancer drug resistance and upregulation of immune checkpoint proteins, both capable of circumventing cytotoxicity against cancer cells by immune cells or drugs. Stem cell-like resistance against chemotherapy and radiotherapy is mediated by upregulation of drug transport pumps, cellular quiescence, enhanced DNA repair abilities, overexpression of multidrug-resistance gene (MDR) and anti-apoptosis mechanisms[13]. Recent studies reported that activation of EMT in carcinoma cells can diminish the response to immunotherapy, mediated by release of TGF-β1, which increases the number of immunosuppressive regulatory T (Treg) cells and suppresses CD8+ cytotoxic T cells and natural killer (NK) cell activity[14]. Also, EMT increases tumoral expression of programmed death ligand-1, capable of conferring resistance to checkpoint blockade-based immunotherapy, thus exacerbating immune evasion[15] (Figure 2).

Metabolic changes Cancer cells are known to reprogram their metabolic circuitries to meet the bioenergetic demands associated with EMT. Apart from their canonical roles, EMT-inducing signals, transcription factors and lncRNAs are shown to enhance glycolysis and fatty acid oxidation (FAO), and suppress oxidative phosphorylation (OXPHOS). Conversely, EMT-inhibiting miRNA can regulate the expression of metabolite transporters and metabolic enzymes, thus inhibiting both glycolysis and FAO. It is now recognised that these dysfunctional metabolic changes can influence EMT[16] (Figure 2). For example, Pyruvate kinase M2, a glycolytic rate-limiting enzyme, have been shown to relieve transcriptional suppression of the gene encoding for E-cadherin, thus promoting EMT in CRC cells[17]. Also, it has been suggested that Acetyl CoA carboxylase 1, a FAO rate limiting enzyme, can be inactivated in breast cancer. This eventually leads to acetylation of EMT-inducing Smad2[18].

Regulation of EMT

EMT is highly regulated and orchestrated to insure the correct spatial and temporal activation of all the participating genes. To date, a wide array of factors has been shown to be involved in carcinogenic EMT and they can be broadly categorized into the following: **EMT inducers** which kickstart the EMT program; **EMT regulators** which orchestrate and direct the progression of EMT; **EMT effectors** which deal with the execution and delivery of the functional consequences of EMT (Figure 3).

EMT inducers

Canonical WNT/β-Catenin Signalling ~~One of the~~Perhaps the best-known inducer~~s~~ of CRC is the near universal activation of the WNT/β-Catenin signalling pathway[19]. Fundamentally, canonical WNT/β-Catenin pathway stops β-Catenin nuclear accumulation by sequestering it into the cytoplasm. Enhanced WNT signalling results in upregulated level of Snail, a core regulator of EMT that represses E-cadherin and promotes migration and local invasion[20,21]. This crosstalk between WNT signalling and Snail then oscillates in a positive feedback loop where overexpression of Snail enhances expression of WNT target genes[22]. Additionally, Glycogen Synthase Kinase 3 beta (GSK3β) is inhibited by enhanced WNT signalling. This allows Slug to avoid destruction by shielding it from GSK3β phosphorylation and β-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TrCP)-mediated ubiquitination. Lastly, accumulation of Snail and Slug also represses E-Cadherin, a major hallmark symbolizing EMT[23].

TGF-β Signalling It has been well established that TGF-β signalling exhibits tumour static effects during early cancer onset[24,25]. but plays a dichotomous role in late stage tumourigenesis[26,27]. Canonical TGF-β Signalling involves heterodimerization of type I and type II receptor serine/threonine kinases (*TGFBR1/2)* upon ligand binding[28]. This propagates a signal via phosphorylation to Smad proteins. Ultimately, cooperation amongst Smad2,3 and 4 with other transcription factors induces gene transcription events which lead to EMT[29]. TGF-β can also induce EMT via a Smad4 independent pathway, mediated by Rho-A which induces expression of Slug and triggers EMT[30,31].

Notch Signalling Notch signalling is an extensive signalling network that was found to be actively involved in CRC EMT, mediated by Notch1 inducing Jagged1 upregulation in CRC. Subsequently, additional Notch receptors like Notch3 are activated in response to this interaction, increasing CD44, Slug and Smad3 expression, eventually leading to EMT[32].

RAS Signalling RAS operates via two main branches of cellular pathways: mitogen-activated protein kinases and phosphoinositide-3 kinase (PI3K) pathways. These pathways are primarily activated by the activation of receptor tyrosine kinases (RTK), but it can also receive crosstalk signals from TGF-β via Smad4 independent path. Trickling down the canonical signalling cascades, it ultimately activates transcription factors like NF-κB, and Snail that are known to promote EMT.

EMT regulators

The transcriptional machinery which regulates EMT can be divided into 3 branches, namely, the SNAIL, ZEB and TWIST families.

SNAIL family SNAIL family of transcription factors takes the form of zinc-finger, all of which bind to a common binding motif known as the E-box[33,34]. Similarly, Snail2 more commonly known as Slug shares the same binding pattern as Snail[35,36], but it should be noted that

there are still molecular differences between the two genes, given their differential spatial distribution[37]. In addition to their primary role in repressing E-cadherin expression, Snail and Slug coordinate with diverse pathways required to activate the expression of mesenchymal genes; vimentin, N-cadherin and fibronectin[38,39].Additionally, overexpression of Slug has been found to induce other EMT-regulators like Zeb1 or Zeb2[40].

ZEB family Zeb1 and Zeb2 bind to regulatory gene motifs at E-boxes and have been rigorously proven to be one of the master regulatory families of EMT[41-43] and are often found to be upregulated in CRC[44,45]. Zeb1/2 regulate EMT directly by binding to E-box on E-cadherin (CDH1) promoter region[46], in a similar fashion to Snail or indirectly through the recruitment of co-repressor C-terminal-binding protein (CtBP)[47]. Both result in downregulation of E-cadherin protein expression. Moreover, Zeb1 is capable of inducing the loss of basal membrane by silencing epithelial BM component Laminin subunit alpha-3 (LAMA3) expression[48]. Concomitantly, upregulation of Zeb1 activates expression of mesenchymal genes like laminin gamma 2 (LAMC2) and urokinase plasminogen activator (uPA) to promote tumour invasiveness[49]. Similarly, hyper expression of Zeb2 also actuates other EMT proteins such as matrix metalloproteinase 9 (MMP-9) and Twist[50].

TWIST *family* Studies have shown that Twist1/2 are overexpressed in a wide array of cancers, including CRC[51,52] and activate the N-cadherin promoter and switch on mesenchymal markers such as N-cadherin[53] and fibronectin[54]. Consequently, this causes a loss of E-cadherin-mediated cell-cell adhesion, thereby promoting EMT.

EMT effectors

Beyond the activation of EMT transcription factors at genomic level, EMT is ultimately executed and delivered by 2 separate dynamic events: (a) downregulation of epithelial adhesive protein E-cadherin, junctional proteins like occludins and claudins, which leads to destabilization of cell adhesion. (b) Gain of mesenchymal protein products like N-cadherin, vimentin and fibronectin[55], which drive cell locomotion and invasion.

Vimentin Aside from the classically described loss and gain of E- and N-cadherin respectively, vimentin has also proven to be an excellent indicator for EMT in CRC[56]. Vimentin is a type III intermediate filament (IF) cytoskeletal protein primarily found in mesenchymal cells[57]. Functionally, vimentin confers resistance and integrity to the cells[58]. Beyond this basic function, it also promotes cell migration by processing mechanical feedbacks and modulating the dynamics of microtubules and the actomyosin network[59]. As such, abnormal expression of vimentin as in CRC undoubtedly promotes CRC invasion.

Fibronectin Fibronectin is an extracellular matrix glycoprotein responsible for cell-adhesion and migration in mesenchymal cells. However, accumulating evidence has pointed out that fibronectin is often implicated in CRC pathogenesis and elevated levels of fibronectin are closely associated with poor CRC prognosis[60,61]. More recently, it has been elucidated that CRC-derived fibronectin extra domain A (EDA) promotes EMT in

surrounding CRC cells via interaction with α9β1 integrin. Although the precise mechanism remains unknown, this eventually leads to upregulation of Snail and vimentin and substantially reduced E-cadherin levels[60].

Use of EMT markers to predict prognosis.

Molecular typing and genotyping are finding an increasing role to guide clinicians in the stratification of different tumour types. Identification of predictive biomarkers allows companion diagnostics to be matched to specific therapies, and prognostic indicators permit treatment intensity to be tailored to risk of tumour recurrence or progression.

There are wealth of data suggesting that individual markers of EMT (eg. loss of E cadherin expression, overexpression of vimentin) may act as predictors for therapeutic and survival outcomes of CRC patients[13,44,61-67]. However, most of these studies of individual markers are relatively modest in size and require independent validation in larger patient cohorts, where they should be tested in combination using multiplexing immunohistochemistry. Multi-marker combination analysis of the altered expression of E-cadherin, β-catenin, vimentin, snail and CD133 in the invasive front of CRC showed stronger correlation with disease-free survival (DFS) (66.2 vs. 84.6 months) and overall survival (OS) (60.8 vs. 77.9 months) than individual protein analysis[13]. Similarly, another study has demonstrated that combining EMT markers like E-cadherin, β-catenin, Snail and Zeb1 improves prognostic performance. In 185 patients with CRC, cytoplasmic S~~s~~nail (HR 1.94 95% confidence interval [CI] 1.15–3.29, $p = 0.012$) and the combined biomarker EMT score (HR 3.86 95% CI 2.17–6.86, $p < 0.001$) were associated with decreased cancer-specific survival[68].

These examples highlight the strength of combinatory analysis in unravelling observations that may be missed when genes are viewed individually. This approach has also proven useful in other cancers apart from CRC. A study comprising of 78 oesophageal squamous cell carcinoma cases demonstrated that the vimentin/E-cadherin ratio was correlated with tumour invasion and can serve as an independent prognostic factor among chemo-naive patients[69]. Likewise, the combined expression pattern of 6 EMT-related proteins (snail-1, vimentin, S100A4, CK19, E-cadherin, β-catenin) predicted shortened disease-free survival in cholangiocarcinoma[70].

Building on these examples, large-scale sequencing technologies has enabled the creation of Consensus Molecular Subtyping (CMS) which comprises four groups with distinctive features of which CMS4 (mesenchymal, 23%), exhibits prominent transforming growth factor–activation, stromal invasion and angiogenesis[71]. Specially, CMS4 classification is associated with EMT features characterised with a distinct drop in miRNAs that regulate tumour suppression via Zeb1 and/or Zeb2. This attribute coupled with the gain of matrix remodelling and TGF-*β* associated signatures likely explained why CMS4 exhibited the worst prognosis amongst the four subtypes, with the worst ~~overall survival~~OS and worst relapse-free survival[72].

Analysis of EMT signatures in liquid biopsies, especially in circulating tumour cells (CTCs), has emerged as another promising prognostic indicator. CTCs characterized by the presence of EMT features with hybrid epithelial and mesenchymal (EM) phenotypes have been associated with increased likelihood of metastasis and worse survival in CRC patients[73] and may also serve as an indicator of therapeutic response[74]. CTCs are currently isolated using the epithelial cell adhesion molecule (EpCAm)-based enrichment technique, but CTC subgroups will obviously not be detected using this technique, given their deletion of this cell surface marker. Thus, a combination of different epithelial and mesenchymal biomarkers might provide a practical solution to reflect a clearer picture of all CTC compartments and therefore refine their prognostic value.

EMT elements as therapeutic targets

Clinical development pathways targeting EMT inducers, regulators or effectors face several challenges due to the plasticity and heterogeneity of the various pathways involved. The spillover effects amongst pathways further permutate the complexity in drugging through EMT. As such, targeting of a single EMT receptor is unlikely to be effective due to the redundant nature of several pathways, whereas perhaps focusing upstream on EMT-TFs as a target could have a more pronounced phenotypic effect. Aside from the technical obstacles in discovering specific molecular inhibitors, the biological features of EMT transformed cells, increased cell mobility, invasiveness, increased metastability and relative chemoresistance present trial design complexities. The vast majority of anticancer agents are antiproliferative, pro-apoptotic and necrotic giving more immediate surrogates of clinical activity like reduction in tumour volume, reduction in the rate of rise of tumour markers or of tumoural metabolic activity. For drugs which seek to inhibit EMT, ~~two~~ three broad therapeutic strategies ~~are in question~~could be applied. Firstly, combination with conventional anticancer drugs to overcome pharmacological resistance in advanced disease~~, using PFS in randomised phase II/III trials.~~ Although chemotherapy forms the backbone of CRC treatment, it is rarely if ever, curative as chemoresistance is all but inevitable. Mechanistically, there are several means through which disruption of cellular functions participating in EMT can confer resistance to 5-Fluorouracil, the most widely used anticancer treatment for CRC. For example, proinflammatory cytokine CCL21 can lead to 5-Fluorouracil resistance through AKT/GSK3β/Snail-induction of P-glycoprotein-1, a MDR protein[75]. Secondly, for use in the adjuvant setting to reduce recurrence, perhaps after resection of metastatic disease, where the event rate is higher than after resection of primary tumours, and where there is no accepted role for post-operative chemotherapy, thus simplifying trial design. Thirdly, but most distant as a therapeutic strategy, one could consider that EMT inhibitors could be developed as chemopreventive agents, if drugs with an appropriate safety profile could be found. Here we summarised in Table 1 on a list of drugs at various stage of clinical trials, capable of targeting EMT markers.

Inducers Several therapeutic strategies against TGF-β are currently under experimental

testing or clinical trial. Among the different pharmacological approaches to block TGF-β signalling there is a particular focus on small molecule inhibitors. Galunisertib (LY2157299), an oral small molecule inhibitor of the TGF-β receptor I kinase, was able to specifically downregulate the phosphorylation of Smad2, thus abrogating activation of the canonical TGF-β pathway[76]. Similarly, LY2109761, a dual kinase inhibitor of TGF-βbeta type I and type II receptors, inhibited TGF-β-mediated activation of Smad and non-Smad pathways in colon adenocarcinoma cells, attenuated cell migration, invasion and tumorigenicity, and further decreased liver metastases and prolonged survival in a metastasis model[77]. The mean survival of LY2109761-treated mice were prolonged to 35.2 days compared with 24.5 days in control mice ($P < 0.001$). TGF-β1-blocking peptides P17 and P144 significantly reduced metastasis to the liver induced by TGF-β1 in a mouse model of CRC[78]. Interestingly, some natural compounds were reported to have antitumorigenic property acting on TGF-β. Resveratrol is extracted from Chinese herbal medicine Polygonum cuspidatum and in vitro assays suggested that resveratrol reduced the rate of lung metastases and hepatic metastases by inhibiting TGF-β1-induced EMT in CRC models[79]. An alternative strategy is monoclonal antibody to target TGF-β. However, the available experimental evidence in CRC is still limited as compared to other tumour types. Fresolimumab (GC-1008) is a human anti-TGF-β monoclonal antibody that neutralizes all isoforms of TGF-β. In a multi-centre phase-I trial, 28 patients with pre-treated malignant melanoma or renal cell carcinoma patients were given GC-1008, and one malignant melanoma patient achieved a partial response, and six had stable disease with a median progression-free survival of 24 weeks. Notably, there was were no drug-related grade 4 or 5 adverse events[80].

Another class of emerging therapy is antisense oligonucleotides (ASOs). ASOs are short (13~25 nucleotides) single-stranded sequences complementary to mRNA sequences of interest, capable of blocking protein translation when bound. Trabedersen (AP 12009) binds specifically to TGF-β2 mRNA. An ongoing phase I/II study used Trabedersen has been used to treat patients with stage IV pancreatic carcinoma, malignant melanoma, or colorectal carcinoma and brain cancers. Encouraging preliminary results were observed on its efficacy: One pancreatic carcinoma (stage IV) patient who received Trabedersen (80 mg/m^2/d), experienced a complete regression of liver metastases and survived past 128 weeks[81]. However, Trabedersen failed to demonstrate increased antitumorigenicity in patients with glioblastoma and anaplastic astrocytoma in a phase II trial[82].

Regorafenib, an oral multitargeted tyrosine kinase inhibitor (TKI) of BRAF, VEGFR-1, -2, -3, KIT, TIE-2, PDGFR-β, FGFR-1, RET and RAF-1, has been approved by FDA for mCRC patients as a last-line therapy. A study has found that regorafenib activates Protein tyrosine phosphatase (PTPase) SH2-domain-containing phosphatase 1 (SHP-1) resulting in the abolishment of EMT-induced invasion and metastasis in CRC[83].

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has been found to resist EMT-related changes, including modifications of β-catenin intracellular localization or vimentin and E-cadherin levels, induced by hypoxia and/or EGF in CRC cell lines[84], although

Despite its potential, our trial of ~~the~~ a COX-2 inhibitor, Rofecoxib in the adjuvant setting for CRC was negative[85].

Cabozantinib is a multi-kinase inhibitor targeting VEGFR, MET, FLT3, c-Kit, and AXL. In CRC, knockdown of AXL resulted in decreased migration and invasion by downregulation of transcription factors required for EMT, including Slug, Twist, and Zeb1, and to increased expression of E-cadherin[86]. A clinical trial ~~is ongoing to investigate~~involving combinational therapy of cabozantinib and panitumumab (an EGFR-targeting monoclonal antibody) in KRAS wild-type CRC (NCT02008383) reported an objective response rate (ORR) of 16%[87]. ~~and final results are not yet available.~~

Regulators The powerful transcriptional machinery controlling proliferation, invasion, and migration make EMT regulators appealing drug discovery targets. As tantalising as they are, there is a profound paucity of drugs against EMT regulators as transcription factors have been widely deemed as “undruggable”. One underlying reason why drugging EMT regulators proves to be a difficult challenge to surmount, is due to the fact (a) transcription is a nuclear event that is not readily accessible to drugs, (b) highly positively charged helical DNA binding interfaces limit drug accessibility, and (c) flat protein–protein interaction surfaces, lacking deep druggable binding pockets[88,89]. ~~There are several tantalising preclinical observations supporting this thesis. Moscatilin, derived from the orchid Dendrobrium loddigesii, has been shown to induce cell cycle arrest and apoptosis in CRC cells. Moscatilin has been further shown to act on AKT/TWIST signalling and suppress migration and metastasis of breast cancer cells. Snail has also been a popular drug target and depletion restored cellular sensitivity to chemotherapy in lung cancer cells. High mobility group A2 (HMGA2) protein was reported to be able to induce EMT by regulating Snail expression. A small-molecule antifungal agent ciclopirox (CPX) has been identified as a novel inhibitor of HMGA2 and it can induce cytotoxicity in CRC through direct interaction with HMGA2.~~

Effectors The loss of E-cadherin is a key feature of EMT, and restoration of its expression could be considered to a promising approach to suppress metastasis. A pilot drug library screen (n=9600) revealed that Methotrexate (MTX) was able to induce a 10-fold increase in E-cadherin expression in CRC cell lines. MTX is not active as a single agent in the treatment of advanced CRC, but these data, which require further validation in more extensive models, possibly suggest a clinical development pathway, using low dose oral MTX following conventional adjuvant chemotherapy to prevent further metastasis, as a quasi-maintenance therapy[90]. Similarly, the ionophore antibiotic salinomycin downregulates the expression of vimentin while increasing the expression of E-cadherin in $CD133^+$ CRC cells and can reverse doxorubicin-induced EMT, restoring chemosensitivity both in vitro and in vivo in hepatocellular cancer xenografts, warranting further clinical investigation[91].

Apart from targeting cells that express active biomarkers of EMT and prevention of EMT induction discussed above, many efforts have also been made to investigate the reversion

of EMT by induction of the reverse mesenchymal-epithelial transition (MET) program. Previous data have indicated that activation of MET in human mammary epithelial cells (HMLE cells) resulted in reduced invasiveness, increased spheroid formation ability and increased sensitivity to therapeutic agents including doxorubicin, paclitaxel, proteasome inhibitors and EGFR inhibitors[92]. Although these findings proved to be promising, there lies a valley of death to be bridged before they can be applied in clinical practice. Particularly, the inactivation of EMT and induction of MET are both necessary for efficient metastatic colonization and outgrowth at the distant site[93]. In this context, therapeutically promoting MET may accelerate metastasis of disseminated cells. Thus, precise therapeutic windows have to be carefully defined.

Beyond EMT Apart from drugging EMT targets directly, exploration has been made into other landscapes to repurpose drugs that can potentially impact EMT status indirectly. Metabolic reprogramming has been gaining traction as an approach for EMT phenotypic switching in cancer cells[16]. Ramesh et al., have recently published a key list of metabolic drugs reported to have EMT-inhbiting features[94]. Many of which have already been approved for cancers or other diseases, indicating that agents with acceptable toxicity profiles and clinical efficacy are available for additional trials.

Discussion

EMT programs are highly orchestrated through a complex network which encompasses numerous regulatory factors of many cellular signalling pathways, with the latter interplaying with each other to form extensive crosstalk. Recent research has provided evidence that the elements driving EMT may be exploited as potentially useful prognostic markers or as drug development targets. However, we have also underlined the challenges of translating these preclinical or early clinical findings into routine practice. These include potential on-target, off-tumour toxicity, given that these mesenchymal markers are widely expressed by non-tumour mesenchymal cells, such as fibroblasts; the regulatory network governing EMT is complex, with significant cross talk and redundancy making identification of a master controller difficult; prevention of EMT induction by interfering with TGF-β signalling requires recognition of its dual effect in CRC, and a balance would need to be found between abrogation of the pro-tumour properties of TGF-β signalling without impairing its tumour suppressor functions in early stages of the disease.

Another obstacle derives from the non-negligible gap between the preclinical models and clinical cancer progression. The induced model of EMT is commonly used in most preclinical studies to mimic the effect of EMT on invasiveness, metastasis and drug resistance. They are also frequently used to assess the efficacy of drug development programs. However, these models may not fully recapitulate the true heterogeneity of a tumour and the transient and reversible plasticity of EMT that is essential for tumour progression is difficult reproduce in these models. Given the current climate of research,

one suggestion to overcome this obstacle will be to eliminate semantic problems in EMT through a levelled interdisciplinary discussion and collaboration. It is, therefore, of interest to highlight the consensus statement drawn up in 2020 by the EMT International Association (TEMTIA), that aims to provide consensual guidelines for EMT research[95]. This will go a long way towards guiding early career scientist pursuing this field as well as benefitting existing researchers in reaching consensus on a panel of robust and validated biomarkers, useful for clinical drug testing. Also, it is worthwhile to take a combinatorial or multi-omics approach, integrating metabolic, proteomic and gene signatures together at single-cell resolution in order to robustly establish biomarkers for EMT, particularly for the elusive pEMT.

This field does represent an interesting clinical development challenge. Most novel anticancer agents are first tested against advanced tumours, to demonstrate tumour shrinkage and improvements in ~~progression free~~PFS and perhaps OS~~overall survival~~ before proceeding to treatment of early-stage disease. ~~If we are to follow the science, as it was, and plan to introduce EMT inhibitors into the clinic~~As such, for these preclinical EMT inhibitors to be taken from bench to bedside, ~~it~~identification of robust and validated biomarkers should take precedence. Although pEMT constitutes to the most malignant form of cancers, it remains elusive in terms of specific biomarkers. Therefore, it is imperative to place focus on this particular aspect before any form of clinical testing becomes a reality. ~~would be logical to consider a trial to prevent metastasis. One possible clinical scenario could therefore be after resection of hepatic metastases or of biomarker defined high risk primary disease.~~

Search strategy and selection criteria

References for this Review were identified by searches of Pubmed using the search terms “epithelial-mesenchymal transition”, “EMT”, “MET”, “pEMT”, “colorectal cancer”, “cancer”, “chemotherapy”, “immunotherapy”, “targeted therapy”, “chemoresistance”, “prognosis”, “consensus molecular subtypes”, “CMS”. No date limits were applied. Papers published in English were reviewed. Additionally, ClinicalTrials.gov was visited during May 2020 to February 2021 for trials of the targeted therapies of interest.

Contributors

N.Z., A.S.N., and D.K. conceptualised the manuscript. N.Z. and A.S.N. did the literature review and wrote the original draft. A.S.N. conceptualised and did the illustrations ~~using Biorender.com~~. N.Z., A.S.N., and D.K. did the revision of the manuscript. S.C., Q.L., Y.L., and D.K. supervised the project.

Declaration of interests

D.K. reports research grant from University of Oxford-Sichuan University Huaxi Joint Centre for Gastrointestinal Cancer; consultation fees from SAB and Indivumed; stock options as Director from Oxford Cancer Biomarkers and Celleron Therapeutics. All other authors declare no competing interests.

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Novel Therapeutic Strategies: Targeting Epithelial-Mesenchymal Transition in Colorectal Cancer

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Abstract

Epithelial-mesenchymal transition (EMT) is a process during which cells lose their epithelial characteristics, for instance apical-basal cell polarity and cell–cell contact, and gain mesenchymal properties, such as increased motility. In colorectal cancer (CRC), EMT plays an important role in tumour progression, metastasis, and drug resistance. There have been accumulating evidence from preclinical and early clinical studies which show that EMT markers may serve as outcome predictors and potential therapeutic targets in CRC. This review decribes the fundamentals of EMT which include the biology of EMT and the newly-minted partial EMT (pEMT), and their associated changes. We have also provided a comprehensive summary of therapeutic compounds capable of targetting EMT markers, ranging from preclinical stage, clinical trials or repurpose potential. Lastly, we have also explored on the potential obstacles that lie ahead of EMT bench to bedside drug development.

Introduction

Colorectal cancer (CRC) is the third most prevalent malignant tumour worldwide, accounting for more than 9% of annual cancer mortality[1]. Although advances have been made in screening, early detection and management of established disease, targeted therapeutic innovations have been relatively sparse. It is therefore widely acknowledged that a deeper understanding of the complex biology of CRC must precede rational drug development.

Epithelial-mesenchymal transition (EMT) was first described in embryogenesis and refers to a cellular reprogramming process in which epithelial cells acquire a mesenchymal phenotype. EMT plays an important role in development, wound healing, and malignant progression in which cancer cells are endowed with properties associated with more aggressive phenotypes[2].

Upon activation of EMT, tumour cells undergo a series of physical changes including tight junction dissolution, disruption of apical–basal polarity, and reorganization of the cytoskeletal architecture, all of which facilitate the dissemination of cells from their primary

site, invasion of surrounding tissues, survival in the general circulation, leading finally to the formation of metastases in distant organs. In addition, recent studies have attributed increased resistance to chemotherapy and immunotherapy to EMT as it promotes interaction with tumour-associated stromal cells, known for their protumorogenic properties[3,4].

EMT-associated changes

EMT is a generic description integrating several processes which share certain common features, but which can vary according to the tissue origin, stromal cell components and environmental cytokines. EMT is multifaceted, often reversible and involves a range of changes in cell biology, gene expression and physiology. Traditionally, EMT is though to be a binary process. However, accumulating evidence has shown otherwise. EMT programming runs on a spectrum; it can range from complete EMT/MET to cells with incomplete suppression of pre-existing epithelial characteristics and incomplete acquisition of mesenchymal ones, a hybrid intermediate stage known as partial EMT (pEMT)[5]. In the intervening years, heterogenous populations with different degree of EMT can be observed within tumours and pEMT has been widely observed across a broad range of cancers. Recent evidence has proven that cancer populations with pEMT have the highest metastatic potential. Interestingly, among these hybrid subpopulations, those with more epithelial features and less mesenchymal conversion have the greatest malignancy and metastatic potential[6,7](Figure 1). It has been reported that patients with primary prostate, breast or lung cancers that exhibit the full range of epithelial-mesenchymal plasticity, that is, transcriptional evidence of both EMT and MET, have the poorest outcomes[8].

Cellular changes Increased motility and migration through extracellular matrix (ECM) is a typical feature of cells undergoing EMT, during which epithelial cell-cell junctions are dissolved, and the integrity of their basement membranes compromised. These changes are accompanied by alterations in cell polarity, cytoskeletal rearrangements, and a switch in expression from mainly keratin to mainly vimentin. Morphologically, this leads to a striking loss of the typical polygonal, cobblestone appearance of epithelia and the emergence of spindle-shaped fibrous cells which express mesenchymal cell markers, eg. neural cadherin (N-cadherin), vimentin and fibronectin and cell-associated matrix metalloproteinase (MMP) activity[9].

Genetic changes A number of highly conserved transcription factors (EMT-TFs) have been identified, such as Snail, Slug (Snail2), Zeb1, Zeb2, and Twist, which are major regulators of EMT programs, functioning in a variety of cell-specific combinations with each another. In addition, a number of microRNAs (miRNAs) as well as non-coding RNAs can regulate, and be regulated by, the key EMT genes and influence EMT programming. Among these non-coding RNAs, miR-200 family and miR-34 family are the most researched (Figure 1). For example, miR-200c targets *Zeb1* to suppress its expression, thus inhibiting migration and invasion of CRC[10]. These interrelationships leads to numerous permutations which call for the need to resolve them for better understanding of

CRC EMT.

Physiological changes Many studies have demonstrated an association between EMT and acquisition of stem cell-like properties, characterised by expression of classical surface markers. CD51, a putative cancer stem cell marker in CRC, has been found to be one of the most frequently expressed markers[11]. CD44- and SOX2-enriched tumorspheres have been detected in TGFβ1-treated CRC cells[12]. These acquisitions confer a range of properties including relative anticancer drug resistance and upregulation of immune checkpoint proteins, both capable of circumventing cytotoxicity against cancer cells by immune cells or drugs. Stem cell-like resistance against chemotherapy and radiotherapy is mediated by upregulation of drug transport pumps, cellular quiescence, enhanced DNA repair abilities, overexpression of multidrug-resistance gene (MDR) and anti-apoptosis mechanisms[13]. Recent studies reported that activation of EMT in carcinoma cells can diminish the response to immunotherapy, mediated by release of TGF-β1, which increases the number of immunosuppressive regulatory T (Treg) cells and suppresses CD8+ cytotoxic T cells and natural killer (NK) cell activity[14]. Also, EMT increases tumoral expression of programmed death ligand-1, capable of conferring resistance to checkpoint blockade-based immunotherapy, thus exacerbating immune evasion[15] (Figure 2).

Metabolic changes Cancer cells are known to reprogram their metabolic circuitries to meet the bioenergetic demands associated with EMT. Apart from their canonical roles, EMT-inducing signals, transcription factors and lncRNAs are shown to enhance glycolysis and fatty acid oxidation (FAO), and suppress oxidative phosphorylation (OXPHOS). Conversely, EMT-inhibiting miRNA can regulate the expression of metabolite transporters and metabolic enzymes, thus inhibiting both glycolysis and FAO. It is now recognised that these dysfunctional metabolic changes can influence EMT[16] (Figure 2). For example, Pyruvate kinase M2, a glycolytic rate-limiting enzyme, have been shown to relieve transcriptional suppression of the gene encoding for E-cadherin, thus promoting EMT in CRC cells[17]. Also, it has been suggested that Acetyl CoA carboxylase 1, a FAO rate limiting enzyme, can be inactivated in breast cancer. This eventually leads to acetylation of EMT-inducing Smad2[18].

Regulation of EMT

EMT is highly regulated and orchestrated to insure the correct spatial and temporal activation of all the participating genes. To date, a wide array of factors has been shown to be involved in carcinogenic EMT and they can be broadly categorized into the following: **EMT inducers** which kickstart the EMT program; **EMT regulators** which orchestrate and direct the progression of EMT; **EMT effectors** which deal with the execution and delivery of the functional consequences of EMT (Figure 3).

EMT inducers

Canonical WNT/β-Catenin Signalling Perhaps the best-known inducer of CRC is the

near universal activation of the WNT/β-Catenin signalling pathway[19]. Fundamentally, canonical WNT/β-Catenin pathway stops β-Catenin nuclear accumulation by sequestering it into the cytoplasm. Enhanced WNT signalling results in upregulated level of Snail, a core regulator of EMT that represses E-cadherin and promotes migration and local invasion[20,21]. This crosstalk between WNT signalling and Snail then oscillates in a positive feedback loop where overexpression of Snail enhances expression of WNT target genes[22]. Additionally, Glycogen Synthase Kinase 3 beta (GSK3β) is inhibited by enhanced WNT signalling. This allows Slug to avoid destruction by shielding it from GSK3β phosphorylation and β-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (β-TrCP)-mediated ubiquitination. Lastly, accumulation of Snail and Slug also represses E-Cadherin, a major hallmark symbolizing EMT[23].

TGF-β Signalling It has been well established that TGF-β signalling exhibits tumour static effects during early cancer onset[24,25]. but plays a dichotomous role in late stage tumourigenesis[26,27]. Canonical TGF-β Signalling involves heterodimerization of type I and type II receptor serine/threonine kinases (*TGFBR1/2)* upon ligand binding[28]. This propagates a signal via phosphorylation to Smad proteins. Ultimately, cooperation amongst Smad2,3 and 4 with other transcription factors induces gene transcription events which lead to EMT[29]. TGF-β can also induce EMT via a Smad4 independent pathway, mediated by Rho-A which induces expression of Slug and triggers EMT[30,31].

Notch Signalling Notch signalling is an extensive signalling network that was found to be actively involved in CRC EMT, mediated by Notch1 inducing Jagged1 upregulation in CRC. Subsequently, additional Notch receptors like Notch3 are activated in response to this interaction, increasing CD44, Slug and Smad3 expression, eventually leading to EMT[32].

RAS Signalling RAS operates via two main branches of cellular pathways: mitogen-activated protein kinases and phosphoinositide-3 kinase (PI3K) pathways. These pathways are primarily activated by the activation of receptor tyrosine kinases (RTK), but it can also receive crosstalk signals from TGF-β via Smad4 independent path. Trickling down the canonical signalling cascades, it ultimately activates transcription factors like NF-κB, and Snail that are known to promote EMT.

EMT regulators

The transcriptional machinery which regulates EMT can be divided into 3 branches, namely, the SNAIL, ZEB and TWIST families.

SNAIL family SNAIL family of transcription factors takes the form of zinc-finger, all of which bind to a common binding motif known as the E-box[33,34]. Similarly, Snail2 more commonly known as Slug shares the same binding pattern as Snail[35,36], but it should be noted that there are still molecular differences between the two genes, given their differential spatial distribution[37]. In addition to their primary role in repressing E-cadherin expression, Snail and Slug coordinate with diverse pathways required to activate the expression of

mesenchymal genes; vimentin, N-cadherin and fibronectin[38,39].Additionally, overexpression of Slug has been found to induce other EMT-regulators like Zeb1 or Zeb2[40].

ZEB family Zeb1 and Zeb2 bind to regulatory gene motifs at E-boxes and have been rigorously proven to be one of the master regulatory families of EMT[41-43] and are often found to be upregulated in CRC[44,45]. Zeb1/2 regulate EMT directly by binding to E-box on E-cadherin (CDH1) promoter region[46], in a similar fashion to Snail or indirectly through the recruitment of co-repressor C-terminal-binding protein (CtBP)[47]. Both result in downregulation of E-cadherin protein expression. Moreover, Zeb1 is capable of inducing the loss of basal membrane by silencing epithelial BM component Laminin subunit alpha-3 (LAMA3) expression[48]. Concomitantly, upregulation of Zeb1 activates expression of mesenchymal genes like laminin gamma 2 (LAMC2) and urokinase plasminogen activator (uPA) to promote tumour invasiveness[49]. Similarly, hyper expression of Zeb2 also actuates other EMT proteins such as matrix metalloproteinase 9 (MMP-9) and Twist[50].

TWIST *family* Studies have shown that Twist1/2 are overexpressed in a wide array of cancers, including CRC[51,52] and activate the N-cadherin promoter and switch on mesenchymal markers such as N-cadherin[53] and fibronectin[54]. Consequently, this causes a loss of E-cadherin-mediated cell-cell adhesion, thereby promoting EMT.

EMT effectors

Beyond the activation of EMT transcription factors at genomic level, EMT is ultimately executed and delivered by 2 separate dynamic events: (a) downregulation of epithelial adhesive protein E-cadherin, junctional proteins like occludins and claudins, which leads to destabilization of cell adhesion. (b) Gain of mesenchymal protein products like N-cadherin, vimentin and fibronectin[55], which drive cell locomotion and invasion.

Vimentin Aside from the classically described loss and gain of E- and N-cadherin respectively, vimentin has also proven to be an excellent indicator for EMT in CRC[56]. Vimentin is a type III intermediate filament (IF) cytoskeletal protein primarily found in mesenchymal cells[57]. Functionally, vimentin confers resistance and integrity to the cells[58]. Beyond this basic function, it also promotes cell migration by processing mechanical feedbacks and modulating the dynamics of microtubules and the actomyosin network[59]. As such, abnormal expression of vimentin as in CRC undoubtedly promotes CRC invasion.

Fibronectin Fibronectin is an extracellular matrix glycoprotein responsible for cell-adhesion and migration in mesenchymal cells. However, accumulating evidence has pointed out that fibronectin is often implicated in CRC pathogenesis and elevated levels of fibronectin are closely associated with poor CRC prognosis[60,61]. More recently, it has been elucidated that CRC-derived fibronectin extra domain A (EDA) promotes EMT in surrounding CRC cells via interaction with α9β1 integrin. Although the precise mechanism remains unknown, this eventually leads to upregulation of Snail and vimentin and substantially reduced E-cadherin levels[60].

Use of EMT markers to predict prognosis.

Molecular typing and genotyping are finding an increasing role to guide clinicians in the stratification of different tumour types. Identification of predictive biomarkers allows companion diagnostics to be matched to specific therapies, and prognostic indicators permit treatment intensity to be tailored to risk of tumour recurrence or progression.

There are wealth of data suggesting that individual markers of EMT (eg. loss of E cadherin expression, overexpression of vimentin) may act as predictors for therapeutic and survival outcomes of CRC patients[13,44,61-67]. However, most of these studies of individual markers are relatively modest in size and require independent validation in larger patient cohorts, where they should be tested in combination using multiplexing immunohistochemistry. Multi-marker combination analysis of the altered expression of E-cadherin, β-catenin, vimentin, snail and CD133 in the invasive front of CRC showed stronger correlation with disease-free survival (DFS) (66.2 vs. 84.6 months) and overall survival (OS) (60.8 vs. 77.9 months) than individual protein analysis[13]. Similarly, another study has demonstrated that combining EMT markers like E-cadherin, β-catenin, Snail and Zeb1 improves prognostic performance. In 185 patients with CRC, cytoplasmic Snail (HR 1.94 95% confidence interval [CI] 1.15–3.29, $p = 0.012$) and the combined biomarker EMT score (HR 3.86 95% CI 2.17–6.86, $p < 0.001$) were associated with decreased cancer-specific survival[68].

These examples highlight the strength of combinatory analysis in unravelling observations that may be missed when genes are viewed individually. This approach has also proven useful in other cancers apart from CRC. A study comprising of 78 oesophageal squamous cell carcinoma cases demonstrated that the vimentin/E-cadherin ratio was correlated with tumour invasion and can serve as an independent prognostic factor among chemo-naive patients[69]. Likewise, the combined expression pattern of 6 EMT-related proteins (snail-1, vimentin, S100A4, CK19, E-cadherin, β-catenin) predicted shortened disease-free survival in cholangiocarcinoma[70].

Building on these examples, large-scale sequencing technologies has enabled the creation of Consensus Molecular Subtyping (CMS) which comprises four groups with distinctive features of which CMS4 (mesenchymal, 23%), exhibits prominent transforming growth factor–activation, stromal invasion and angiogenesis[71]. Specially, CMS4 classification is associated with EMT features characterised with a distinct drop in miRNAs that regulate tumour suppression via Zeb1 and/or Zeb2. This attribute coupled with the gain of matrix remodelling and TGF-*β* associated signatures likely explained why CMS4 exhibited the worst prognosis amongst the four subtypes, with the worst OS and worst relapse-free survival[72].

Analysis of EMT signatures in liquid biopsies, especially in circulating tumour cells (CTCs), has emerged as another promising prognostic indicator. CTCs characterized by the presence of EMT features with hybrid epithelial and mesenchymal (EM) phenotypes have

been associated with increased likelihood of metastasis and worse survival in CRC patients[73] and may also serve as an indicator of therapeutic response[74]. CTCs are currently isolated using the epithelial cell adhesion molecule (EpCAm)-based enrichment technique, but CTC subgroups will obviously not be detected using this technique, given their deletion of this cell surface marker. Thus, a combination of different epithelial and mesenchymal biomarkers might provide a practical solution to reflect a clearer picture of all CTC compartments and therefore refine their prognostic value.

EMT elements as therapeutic targets

Clinical development pathways targeting EMT inducers, regulators or effectors face several challenges due to the plasticity and heterogeneity of the various pathways involved. The spillover effects amongst pathways further permutate the complexity in drugging through EMT. As such, targeting of a single EMT receptor is unlikely to be effective due to the redundant nature of several pathways, whereas perhaps focusing upstream on EMT-TFs as a target could have a more pronounced phenotypic effect. Aside from the technical obstacles in discovering specific molecular inhibitors, the biological features of EMT transformed cells, increased cell mobility, invasiveness, increased metastability and relative chemoresistance present trial design complexities. The vast majority of anticancer agents are antiproliferative, pro-apoptotic and necrotic giving more immediate surrogates of clinical activity like reduction in tumour volume, reduction in the rate of rise of tumour markers or of tumoural metabolic activity. For drugs which seek to inhibit EMT, three broad therapeutic strategies could be applied. Firstly, combination with conventional anticancer drugs to overcome pharmacological resistance in advanced disease. Althoughchemotherapy forms the backbone of CRC treatment, it is rarely if ever, curative as chemoresistance is all but inevitable. Mechanistically, there are several means through which disruption of cellular functions participating in EMT can confer resistance to 5-Fluorouracil, the most widely used anticancer treatment for CRC. For example, proinflammatory cytokine CCL21 can lead to 5-Fluorouracil resistance through AKT/GSK3β/Snail-induction of P-glycoprotein-1, a MDR protein[75]. Secondly, for use in the adjuvant setting to reduce recurrence, perhaps after resection of metastatic disease, where the event rate is higher than after resection of primary tumours, and where there is no accepted role for post-operative chemotherapy, thus simplifying trial design. Thirdly, but most distant as a therapeutic strategy, one could consider that EMT inhibitors could be developed as chemopreventive agents, if drugs with an appropriate safety profile could be found. Here we summarised in Table 1 on a list of drugs at various stage of clinical trials, capable of targeting EMT markers.

Inducers Several therapeutic strategies against TGF-β are currently under experimental testing or clinical trial. Among the different pharmacological approaches to block TGF-β signalling there is a particular focus on small molecule inhibitors. Galunisertib (LY2157299), an oral small molecule inhibitor of the TGF-β receptor I kinase, was able to specifically downregulate the phosphorylation of Smad2, thus abrogating activation of the canonical TGF-β pathway[76]. Similarly, LY2109761, a dual kinase inhibitor of TGF-β type I and type II

receptors, inhibited TGF-β-mediated activation of Smad and non-Smad pathways in colon adenocarcinoma cells, attenuated cell migration, invasion and tumorigenicity, and further decreased liver metastases and prolonged survival in a metastasis model[77]. The mean survival of LY2109761-treated mice were prolonged to 35.2 days compared with 24.5 days in control mice ($P < 0.001$). TGF-β1-blocking peptides P17 and P144 significantly reduced metastasis to the liver induced by TGF-β1 in a mouse model of CRC[78]. Interestingly, some natural compounds were reported to have antitumorigenic property acting on TGF-β. Resveratrol is extracted from Chinese herbal medicine Polygonum cuspidatum and in vitro assays suggested that resveratrol reduced the rate of lung metastases and hepatic metastases by inhibiting TGF-β1-induced EMT in CRC models[79]. An alternative strategy is monoclonal antibody to target TGF-β. However, the available experimental evidence in CRC is still limited as compared to other tumour types. Fresolimumab (GC-1008) is a human anti-TGF-β monoclonal antibody that neutralizes all isoforms of TGF-β. In a multi-centre phase-I trial, 28 patients with pre-treated malignant melanoma or renal cell carcinoma patients were given GC-1008, and one malignant melanoma patient achieved a partial response, and six had stable disease with a median progression-free survival of 24 weeks. Notably,there were no drug-related grade 4 or 5 adverse events[80].

Another class of emerging therapy is antisense oligonucleotides (ASOs). ASOs are short (13~25 nucleotides) single-stranded sequences complementary to mRNA sequences of interest, capable of blocking protein translation when bound. Trabedersen (AP 12009) binds specifically to TGF-β2 mRNA. Trabedersen has been used to treat patients with stage IV pancreatic carcinoma, malignant melanoma, colorectal carcinoma and brain cancers. Encouraging preliminary results were observed on its efficacy: One pancreatic carcinoma (stage IV) patient who received Trabedersen (80 mg/m^2/d), experienced a complete regression of liver metastases and survived past 128 weeks[81]. However, Trabedersen failed to demonstrate increased antitumorigenicity in patients with glioblastoma and anaplastic astrocytoma in a phase II trial[82].

Regorafenib, an oral multitargeted tyrosine kinase inhibitor (TKI) of BRAF, VEGFR-1, -2, -3, KIT, TIE-2, PDGFR-β, FGFR-1, RET and RAF-1, has been approved by FDA for mCRC patients as a last-line therapy. A study has found that regorafenib activates Protein tyrosine phosphatase (PTPase) SH2-domain-containing phosphatase 1 (SHP-1) resulting in the abolishment of EMT-induced invasion and metastasis in CRC[83].

Celecoxib, a selective cyclooxygenase-2 (COX-2) inhibitor, has been found to resist EMT-related changes, including modifications of β-catenin intracellular localization or vimentin and E-cadherin levels, induced by hypoxia and/or EGF in CRC cell lines[84] Despite its potential, our trial of a COX-2 inhibitor, Rofecoxib in the adjuvant setting for CRC was negative[85].

Cabozantinib is a multi-kinase inhibitor targeting VEGFR, MET, FLT3, c-Kit, and AXL. In CRC, knockdown of AXL resulted in decreased migration and invasion by downregulation of transcription factors required for EMT, including Slug, Twist, and Zeb1, and to increased

expression of E-cadherin[86]. A clinical trial involving combinational therapy of cabozantinib and panitumumab (an EGFR-targeting monoclonal antibody) in KRAS wild-type CRC (NCT02008383) reported an objective response rate (ORR) of 16%[87].

Regulators The powerful transcriptional machinery controlling proliferation, invasion, and migration make EMT regulators appealing drug discovery targets. As tantalising as they are, there is a profound paucity of drugs against EMT regulators as transcription factors have been widely deemed as “undruggable”. One underlying reason why drugging EMT regulators proves to be a difficult challenge to surmount, is due to the fact (a) transcription is a nuclear event that is not readily accessible to drugs, (b) highly positively charged helical DNA binding interfaces limit drug accessibility, and (c) flat protein–protein interaction surfaces, lacking deep druggable binding pockets[88,89].

Effectors The loss of E-cadherin is a key feature of EMT, and restoration of its expression could be considered to a promising approach to suppress metastasis. A pilot drug library screen (n=9600) revealed that Methotrexate (MTX) was able to induce a 10-fold increase in E-cadherin expression in CRC cell lines. MTX is not active as a single agent in the treatment of advanced CRC, but these data, which require further validation in more extensive models, possibly suggest a clinical development pathway, using low dose oral MTX following conventional adjuvant chemotherapy to prevent further metastasis, as a quasi-maintenance therapy[90]. Similarly, the ionophore antibiotic salinomycin downregulates the expression of vimentin while increasing the expression of E-cadherin in $CD133^+$ CRC cells and can reverse doxorubicin-induced EMT, restoring chemosensitivity both in vitro and in vivo in hepatocellular cancer xenografts, warranting further clinical investigation[91].

Apart from targeting cells that express active biomarkers of EMT and prevention of EMT induction discussed above, many efforts have also been made to investigate the reversion of EMT by induction of the reverse mesenchymal-epithelial transition (MET) program. Previous data have indicated that activation of MET in human mammary epithelial cells (HMLE cells) resulted in reduced invasiveness, increased spheroid formation ability and increased sensitivity to therapeutic agents including doxorubicin, paclitaxel, proteasome inhibitors and EGFR inhibitors[92]. Although these findings proved to be promising, there lies a valley of death to be bridged before they can be applied in clinical practice. Particularly, the inactivation of EMT and induction of MET are both necessary for efficient metastatic colonization and outgrowth at the distant site[93]. In this context, therapeutically promoting MET may accelerate metastasis of disseminated cells. Thus, precise therapeutic windows have to be carefully defined.

Beyond EMT Apart from drugging EMT targets directly, exploration has been made into other landscapes to repurpose drugs that can potentially impact EMT status indirectly. Metabolic reprogramming has been gaining traction as an approach for EMT phenotypic switching in cancer cells[16]. Ramesh et al., have recently published a key list of metabolic drugs reported to have EMT-inhbiting features[94]. Many of which have already been

approved for cancers or other diseases, indicating that agents with acceptable toxicity profiles and clinical efficacy are available for additional trials.

Discussion

EMT programs are highly orchestrated through a complex network which encompasses numerous regulatory factors of many cellular signalling pathways, with the latter interplaying with each other to form extensive crosstalk. Recent research has provided evidence that the elements driving EMT may be exploited as potentially useful prognostic markers or as drug development targets. However, we have also underlined the challenges of translating these preclinical or early clinical findings into routine practice. These include potential on-target, off-tumour toxicity, given that these mesenchymal markers are widely expressed by non-tumour mesenchymal cells, such as fibroblasts; the regulatory network governing EMT is complex, with significant cross talk and redundancy making identification of a master controller difficult; prevention of EMT induction by interfering with TGF-β signalling requires recognition of its dual effect in CRC, and a balance would need to be found between abrogation of the pro-tumour properties of TGF-β signalling without impairing its tumour suppressor functions in early stages of the disease.

Another obstacle derives from the non-negligible gap between the preclinical models and clinical cancer progression. The induced model of EMT is commonly used in most preclinical studies to mimic the effect of EMT on invasiveness, metastasis and drug resistance. They are also frequently used to assess the efficacy of drug development programs. However, these models may not fully recapitulate the true heterogeneity of a tumour and the transient and reversible plasticity of EMT that is essential for tumour progression is difficult reproduce in these models. Given the current climate of research, one suggestion to overcome this obstacle will be to eliminate semantic problems in EMT through a levelled interdisciplinary discussion and collaboration. It is, therefore, of interest to highlight the consensus statement drawn up in 2020 by the EMT International Association (TEMTIA), that aims to provide consensual guidelines for EMT research[95]. This will go a long way towards guiding early career scientist pursuing this field as well as benefitting existing researchers in reaching consensus on a panel of robust and validated biomarkers, useful for clinical drug testing. Also, it is worthwhile to take a combinatorial or multi-omics approach, integrating metabolic, proteomic and gene signatures together at single-cell resolution in order to robustly establish biomarkers for EMT, particularly for the elusive pEMT.

This field does represent an interesting clinical development challenge. Most novel anticancer agents are first tested against advanced tumours, to demonstrate tumour shrinkage and improvements in PFS and perhaps OSbefore proceeding to treatment of early-stage disease. As such, for these preclinical EMT inhibitors to be taken from bench to bedside, identification of robust and validated biomarkers should take precedence. Although pEMT constitutes to the most malignant form of cancers, it remains elusive in terms of specific biomarkers. Therefore, it is imperative to place focus on this particular

aspect before any form of clinical testing becomes a reality.

Search strategy and selection criteria
References for this Review were identified by searches of Pubmed using the search terms “epithelial-mesenchymal transition”, “EMT”, “MET”, “pEMT”, “colorectal cancer”, “cancer”, “chemotherapy”, “immunotherapy”, “targeted therapy”, “chemoresistance”, “prognosis”, “consensus molecular subtypes”, “CMS”. No date limits were applied. Papers published in English were reviewed. Additionally, ClinicalTrials.gov was visited during May 2020 to February 2021 for trials of the targeted therapies of interest.

Contributors
N.Z., A.S.N., and D.K. conceptualised the manuscript. N.Z. and A.S.N. did the literature review and wrote the original draft. A.S.N. conceptualised and did the illustrations. N.Z., A.S.N., and D.K. did the revision of the manuscript. S.C., Q.L., Y.L., and D.K. supervised the project.

Declaration of interests
D.K. reports research grant from University of Oxford-Sichuan University Huaxi Joint Centre for Gastrointestinal Cancer; consultation fees from SAB and Indivumed; stock options as Director from Oxford Cancer Biomarkers and Celleron Therapeutics. All other authors declare no competing interests.

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Reviewers' comments:

Reviewer #1: This is a very well written and a comprehensive review focused on the potential of EMT as a target in advanced tumors. The structure and the information included are suitable and accessible for LO readers.
We thank the reviewer for the valuable inputs and for finding our work a well written one.

1 - I would recommend to modify table 1 in order to include the clinical trials conducted to date with EMT targeted agents and the tumor types included.
We have amended the table as suggested by the reviewer and provided corresponding clinical trial identifiers.

2 - I would also suggest to be cautious with the proposal about a trial in the perioperative setting. As it is very well described by the authors, the efficacy of these agents have not been proved and the toxicity profile is a particular issue to consider. Additionally, the interplay between EMT and other key questions in the adjuvant setting like the role of ctDNA or immune signatures needs to be established.
This is an important point and we have modified the discussion to blend in with a point highlighted by reviewer #2 that is to identify robust biomarkers before any clinical drug test can be taken forward.

Reviewer #2: Zhang et al. review basic and clinical aspect of EMT, mostly in the context of colorectal cancer. It is overall a good piece of work, with a good potential to improve the knowledge of the clinical scientists in the EMT world, and therefore the effort is appreciable. There are, however, a number of issues that could be fixed to improve and update the work, as follows:
We thank the reviewer for the valuable inputs and for finding potential in our work.

1) While it is perfectly understandable the need to simplify, schematize and somehow reduce the topic to basic understandable terms, it should also not be oversimplified, especially when this might give the readers some incorrect information. The EMT field went recently through a significant revolution, based on intense debate in the last 10+ years over conflicting data, and now the whole new concept of partial/hybrid EMT has been quite accepted and formalized. The idea, proven by several fundamental papers from 2018 and 2019, is that pEMT cells are those with the highest tumor-initiating and metastasis-initiating capacity (one could see the works by Pasthushenho et al in Nature 2018, but there are many others that could be acknowledged at a similar level). While this concept is delivered in the first part of the manuscript, I think it should be given more importance in the paper, as well as in the figures (Figure 1), as the old-fashioned E. vs M. dualistic model can no longer be supported. See also Williams at al. Nature Reviews Cancer 2019.
We concur with this point made by the reviewer and have edited our manuscript and figure. As reviewer #2 will see in the revised manuscript, we have placed emphasis in pEMT in text and figure, providing readers on the implications of pEMT in cancer malignancy.

2) The mention of CD44highCD24low surface markers in page 2 is confusing, as this is (probably) related to breast cancer. This should be specified and maybe more details on CRC EMT and CSC biomarkers should be given.
We have taken note about the point raised by the reviewer. Indeed, to spotlight CRC, we have identified CSC biomarkers relevant to CRC and have replaced accordingly.

3) Metabolism should be included in the "EMT elements as therapeutic" section, and some space should be dedicated to the potential use of metabolic inhibitors to reduce the EMT lethal effects, citing the work by Ramesh et al. Trends in Cancer 2020, and including more basic examples of that promising approach. Also, an interesting approach on how to integrate metabolism and EMT in the therapeutic decision-making effort has also been recently conceptually introduced by Jia et al. British Journal of Cancer 2021.
Metabolism has been gaining traction in recent years and we think that metabolic reprograming should be highlighted as a therapeutic avenue. As such, we have supplemented information on metabolic changes associated with EMT and explained how existing metabolic drugs can be repurposed to reduce EMT, citing Ramesh, et al.

4) One more reason why targeting EMT regulators may not represent an easy therapeutical target for blocking EMT directly, is due to the fact that they are mainly transcription factors, which are notoriously hard to drug. This fact should be mentioned with relevant literature. I can think for instance Dang et al. Nat Rev Cancer 2017.
This is an important point and we have modified the manuscript to provide explanation behind the paucity of drugs targeting transcription factors.

5) Unfortunately the manuscript I have for review lacks line and page numbering, but in the "Regulators" section (at what could be page 8) a clear link to EMT of the article chosen is missing. While Snail is a TF with EMT-regulating properties, we cannot be sure that the observed effect are in fact due to a specific EMT blockage (wb in the referenced paper are of low quality and not clear interpretation, and IF data are missing). I would suggest the authors to better select their sources and make sure that the information are actually robust enough to be further disseminated.
We concur with the reviewer and have amended the section to avoid conveying the wrong message to readers.

6) Along the same line, a reference should be made to the recent consensus paper: Guidelines and definitions for research on epithelial-mesenchymal transition, recently published in Nature Reviews Mol Cell Biol by Yang et al. This could be also important for anyone that wants to start EMT research from other molecular oncology fields.
We thank the reviewer for providing this important reference. We find this reference extremely applicable in providing groundwork and platform to level out this field to allow interdisciplinary discussions and collaboration. This ties in nicely with the next point raised by the reviewer. Essentially, this consensus statement works towards addressing confounds in the field and can better encourage interdisciplinary research to address the lack of models and to investigate heterogeneity of the disease.

7) I liked and agreed with the points raised in the Discussion. However, I would prefer that also some possible solutions are offered for the obstacles pointed out. For instance, regarding the lack of models to recapitulate and investigate the heterogeneity of the disease, what action could be taken? Similarly, in the following period it needs to be stressed that the clinical testing on EMT-targeting drugs can became reality only in the presence of robust and validated biomarkers (for instance: we need to identify pEMT-specific biomarkers!).
We have tied in point 6 as a platform to provide cross fertilisation of ideas among EMT researchers. We think that identifying biomarkers of pEMT based on a single aspect of biology would be difficult given how protein expression is rarely black or white. As such, we have suggested to combine molecular events from proteome to genetic to metabolome to construct a clearer picture as to what constitute to pEMT.

8) One additional important aspect that I think goes overlooked in this work is the strong impact of EMT in determining chemotherapy resistance. This is also one of the EMT features most consistently reported in different works and among different cancer types, and one of the most interesting in terms of therapeutic implications. What is the situation in CRCs? What are the opportunities associated with it? I think this should not be missing in a Review focused on clinical oncology.
This is an important point raised by the reviewer and we have been consistently keeping it in mind as reflected in the text and figure. But we have since expanded on the situation in CRC; explaining how EMT is associated to chemoresistance against conventional CRC chemotherapy like 5-FU and how developing EMT inhibitors can achieve other positive impact such as chemoprevention, if drugs with an appropriate safety profile could be found.

Editorial comments:

1. Please provide: one preferred degree qualification per author and indicate any full professors; affiliation details (department, institute, city, state, country) for each author; full institutional correspondence address for corresponding author.

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9. Figure legends should be a maximum of 30 words.
   Checked and confirmed.

10. The review should have a maximum of 75 references, if possible.
    As there are many important points raised by the reviewers that we think would be beneficial to address directly in the manuscript, we did not manage to keep it within 75 references in the revised version.

11. The summary can have 150-200 words; please consider increase your summary to have a fully summary of your review.
    We have beefed up the summary.

12. "Search strategy and selection criteria": this should state clearly the sources (databases, journals, or book reference lists, etc) of the material covered and the criteria used to include or exclude studies. Please state which search terms, languages and date ranges were used.
    Checked and confirmed.

13. The tables are currently supplied in an uneditable format. Please supply tables as separate Word files (not excel or fdf/pdf). Each row of data should be in a separate line. Please ensure that rows and columns are not tabbed; data should be entered in cell form.
    Table has been updated and will be supplied as a separate word document.

14. Please state whether any authors are employed by NIH.
    NIL.

Figure

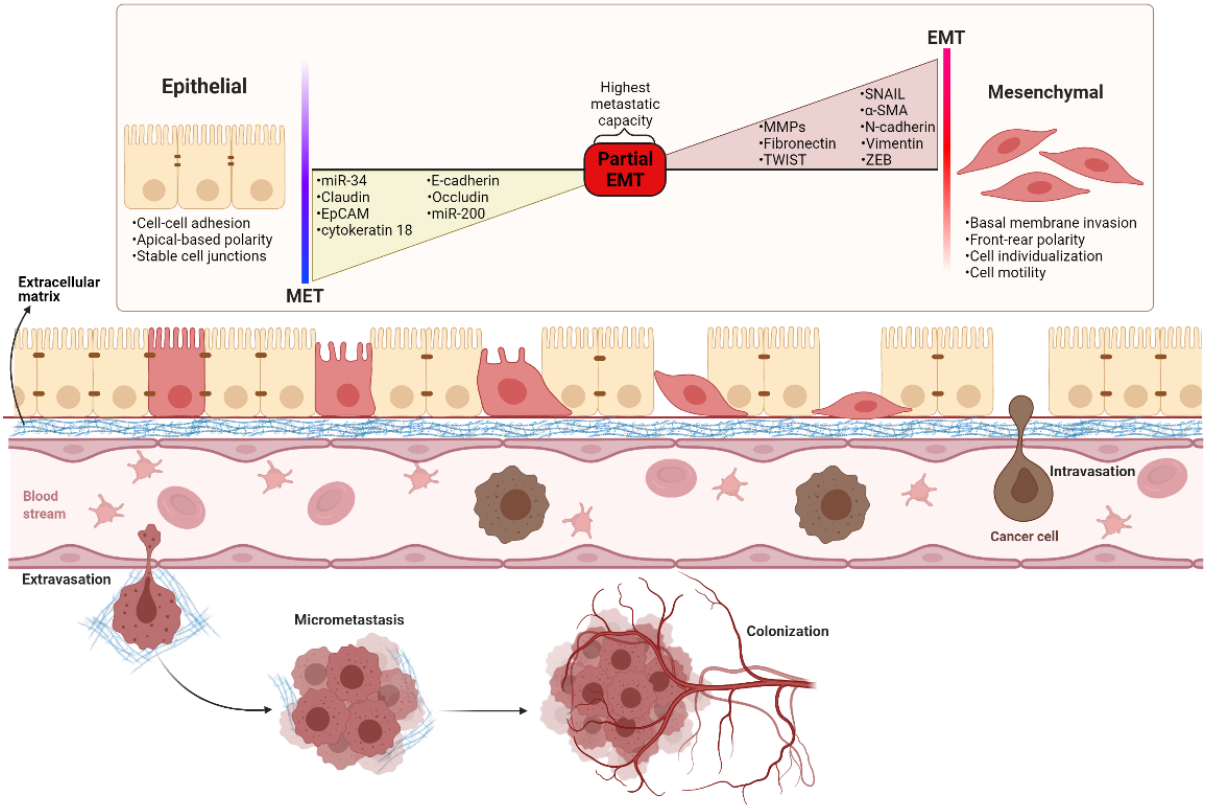

Figure 1: Overview of CRC epithelial mesenchymal transition (EMT) and mesenchymal epithelial transition (MET). EMT and MET are non-binary reversible processes that emphasize on the plasticity of cell to transit along these two states.

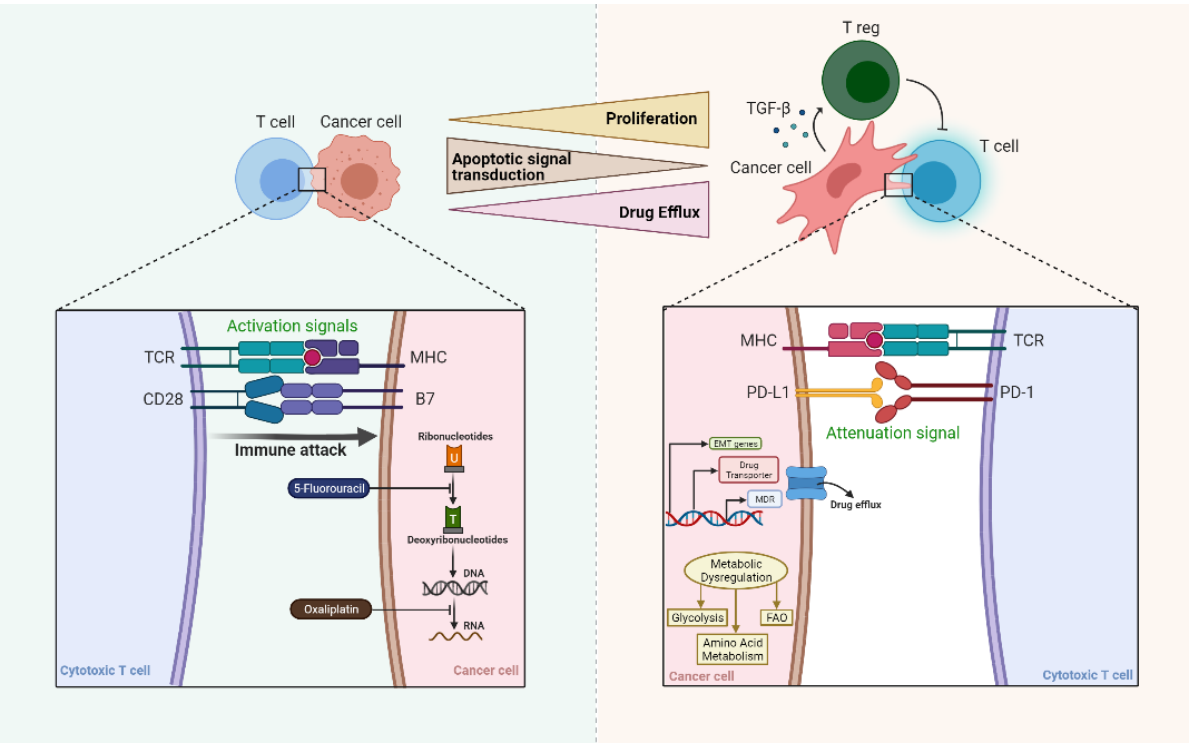

Figure 2: EMT-associated changes. EMT is associated with changes in various aspects; (a) cellular, (b) genetic, (c) physiological, and (d) metabolism. Resultantly, these changes confer CRC with chemoresistance, ability to withstand apoptosis, and support proliferation and invasion.

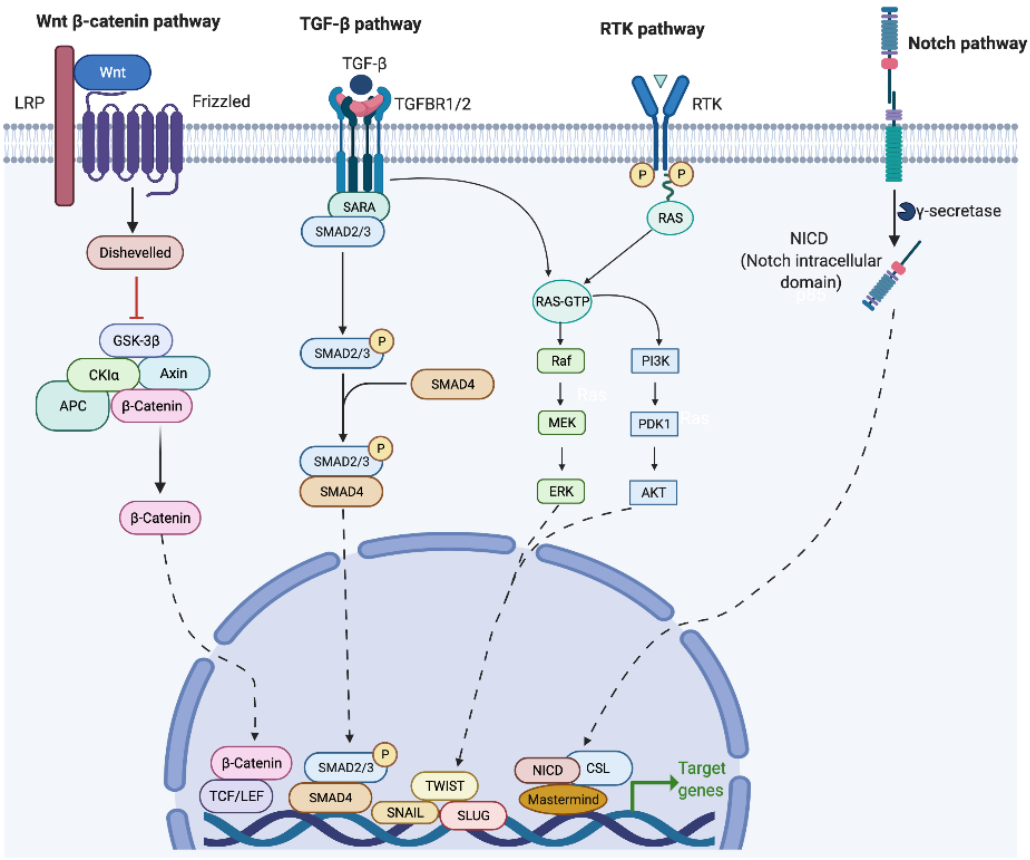

Figure 3: Overview of EMT regulation.

Table

Class	Name	Mechanism of Action
	Galunisertib	TGF-βRI inhibitor
	Vactosertib	TGF-βRI inhibitor
	Resveratrol	TGF-β1 inhibitor
	Baicalin	TGF-β1 inhibitor
	AVID-200	TGF-β inhibitor
	Trabedersen	TGF-β2 mRNA inhibitor
	Regorafenib	RTK inhibitor
	NIS-793	Anti-TGF-β antibody
	Fresolimumab	Anti-TGF-β antibody
	SAR-439459	Anti-TGF-β antibody
	IMC-TR1	Anti-TGF-βRII antibody

Inducers	Celecoxib	COX-2 inhibitor
	Panitumumab	Anti-EGFR antibody
	Simotinib hydrochloride	EGFR inhibitor
	GSK-690693	AKT inhibitor
	Ipatasertib	AKT inhibitor
	Miransertib	AKT inhibitor
	ARQ 751	pan-AKT inhibitor

	Tegavivint	β-catenin inhibitor
	Cabozantinib	RTK inhibitor
Regulators	Metformin	Snail/Twist inhibitor
	MRX34	Snail Inhibitor
	Moscatilin	AKT/Twist inhibitor
Effectors	Ciclopirox	HGM2 inhbitor
	Methotrexate	DHFR inhibitor/ E-cadherin inhibitor
	Salinomycin	Lysosomal iron sequestration

Tumour Type	FDA Approval
Breast, Hepatocellular, Prostate, Carcinosarcoma, Ovarian, Rectal, Gliobastoma, Colorectal & Nasopharyngeal Cancer	Phase I/II
Gastric, Pancreatic, Colorectal, Lung (NSCLC), Urothelial & Myeloma	Phase I/II
Colorectal, Hepatic, Neuroendocrine, Breast, Myeloma & Melanoma	N/A
Colorectal Cancer	Preclinical
Malignant Solid Tumour	Phase I
Pancreatic, Melanoma, Colorectal, Brain Cancer	N/A
Colorectal, Gastrointestinal & Hepatocellular Cancer	Approved
Breast, Lung, Hepatocellular, Colorectal, Pancreatic & Renal cancer	Phase I
Lung Cancer	Phase I/II
Malignant Solid Tumour, Neoplasms & Advance Liver Cancer	Phase I/II
Neoplasm and Tumour	Phase I

Bladder, Colorectal, Pancreatic, Fallopian Tube, Peritoneal Cavity, Recurrent Ovarian Epithelial, Endometrium, Oropharyngeal , Glioma, Breast, Hepatocellular, Prostate, Medulloblastoma, Ependymoma, ATRT, Ewing's Sarcoma	Approved for pain and inflammation Phase I/II/III
Colorectal Cancer	Approved
Non-small Cell Lung Cancer	N/A
Solid Tumour	N/A
Breast, Solid Tumour, Lung, Prostate, Ovarian, Glioma, Gastric & Bladder Cancer	Phase I/II/III
Solid Tumour, Ovarian, Endometrial	N/A
Solid Tumour	N/A

Desmoid Tumour	Phase I
Renal, Thyroid & Hepatic Cancer	Approved
Breast, Colon, Prostate, Pancreatic Cancer	Approved for diabetes Phase I/II/III
SCLC, Lymphoma, Melanoma, Multiple Myeloma, Renal Cell Carcinoma, NSCLC & Primary Liver Cancer	Phase I (Terminated)
Breast Cancer	Preclinical
Advanced Solid Tumour	Approved for antifungal use Phase I
Head and Neck, Glioma, Lung, Bladder & Breast Cancer	Approved for rheumatoid arthritis
Colorectal Cancer	Approved for poultry supplement Preclinical

Table 1: Classes of drugs targeting EMT.

Identifier
NCT02672475
NCT02906397
NCT02178358
NCT02452008
NCT03206177
NCT02688712
NCT01582269
NCT01682187
NCT04031872
NCT04605562
NCT03698825
NCT04656002
NCT04258072
NCT03666832
NCT03724851
NCT03732274
NCT04064190
NCT03143985
NCT00256334
NCT02261844
NCT01476592
NCT03482401
NCT00920556
NCT01107665
N/A
NCT03834662
NCT00844064
NCT00761280
NCT00431561
N/A
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NCT04390763
NCT02581787
NCT03192345
NCT04729725
NCT04524871
NCT01646203

NCT02885974
NCT03645187
NCT03638297
NCT01150045
NCT03026140
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NCT03498326
NCT00538031
NCT03896113
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NCT03184493
NCT00268476
NCT04201561
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NCT02770378
NCT03878524
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NCT04341259
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