

REVIEW



EMT signaling: potential contribution of CRISPR/Cas gene editing

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Abstract

Epithelial to mesenchymal transition (EMT) is a complex plastic and reversible cellular process that has critical roles in diverse physiological and pathological phenomena. EMT is involved in embryonic development, organogenesis and tissue repair, as well as in fibrosis, cancer metastasis and drug resistance. In recent years, the ability to edit the genome using the clustered regularly interspaced palindromic repeats (CRISPR) and associated protein (Cas) system has greatly contributed to identify or validate critical genes in pathway signaling. This review delineates the complex EMT networks and discusses recent studies that have used CRISPR/Cas technology to further advance our understanding of the EMT process.

Keywords Epithelial–mesenchymal transition · CRISPR · Gene editing · Cancer

Introduction

Epithelial to mesenchymal transition (EMT) defines a complex cascade of events through which epithelial cells lose their apico-basal polarity and acquire mesenchymal characteristics leading to a fibroblastic-like morphology with a front-rear polarity. This highly plastic and dynamic

phenomenon is involved in several biological processes including embryonic development and organogenesis, induction of pluripotency, tissue repair, wound healing, as well as pathological conditions including fibrosis, acquisition of stem cell properties by cancer cells, cancer progression and metastasis [1–3]. Indeed, during the EMT program, cells enhance their migratory and invasive properties and often

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express N-cadherin at the expense of E-cadherin expression, the so-called “Cadherin Switch”. This process has important implications in the acquisition of motility by reducing intercellular adhesion strength [4, 5]. EMT is primarily regulated by microenvironmental signals such as growth factors, cytokines, pH and hypoxia. While EMT has been defined since 1968, only recent studies demonstrated that it also plays a critical role in the progression of carcinoma and resistance through inducing stem cell properties, preventing apoptosis and senescence, and contributing to immunosuppression [2, 6–8].

Distinct populations of carcinoma cells coexist in tumors. Most of them belong to the active proliferative pool showing epithelial features, while some cells initiate the EMT program and transit to a mesenchymal status [9]. Moreover, the EMT program is often activated in a reversible way allowing carcinoma cells to revert via the inverse process called mesenchymal to epithelial transition (MET) program to an epithelial status during expansion in metastatic sites [10]. Cells that underwent the EMT program, becoming mesenchymal-like, are able to invade into adjacent tissues and intravasate. Once in the blood or lymphatic flow, they cooperate with non-EMT cells, expressing epithelial markers, for extravasation and colonization in distant organs [11]. Both EMT and non-EMT cells are able to survive in the circulation especially when forming microemboli with platelets [12]. However, single cells that have undergone EMT program may fail to arrest in a secondary site [11, 13, 14]. In addition extravasation can be facilitated by the presence of neutrophils [15]. Therefore, circulating tumor cells (CTCs) demonstrate a dynamic phenotype, however, only cells that have acquired the capability of self-renewal can be defined as cancer stem cells (CSCs) or tumor initiating cells (TICs). These cells are able to form secondary tumors, and often show enhanced chemoresistance. Once extravasated, carcinoma cells form micrometastases and eventually re-express epithelial markers, through MET, to initiate growth and develop into clinically detectable macrometastases [16, 17]. With our enhanced understanding of EMT, this process is now considered as a continuum, with cells exhibiting a spectrum of phenotypes between the epithelial and mesenchymal states [2].

Microenvironmental factors regulating EMT in carcinoma

Inside the core of solid tumors, impaired angiogenesis and dysregulated growth rapidly result in the development of hypoxic conditions that in turn promote the anaerobic glycolytic metabolism causing increased lactic acid production and subsequent acidification of extracellular tumor microenvironment [18–20]. Indeed, two main elements influencing

EMT are hypoxia and acidity. Hypoxia is reported to be responsible for the overexpression of mesenchymal markers such as Vimentin and N-cadherin, and downregulation of epithelial markers such as E-cadherin. This is accompanied by an increase in the invasive and migratory capacity of cancer cells [21]. Hypoxia-inducible factor-1 (HIF-1) is commonly involved in the regulation of the transcription of various target genes in response to hypoxia, and consequential cellular processes. Moreover, Twist, which is also reported to be a downstream target of HIF-1, has a critical role in EMT, regulating several pathways associated with induction of the metastatic phenotype [22]. HIF-1 can also directly regulate the expression of transcription factors implicated in stem cell self-renewal and multipotency and induce the expression of stemness markers in cancer cells [23]. Tumor microenvironmental hypoxia can also promote EMT induction via regulating intracellular messengers; for instance, hypoxic promotion of EMT in breast cancer cells via regulation of cellular reactive oxygen species (ROS) [24] and intracellular calcium signaling [25]. Indeed, HIF-1 and calcium reciprocally interact to promote cancer progression [26]. Another main contributor to EMT in cancer, which is a direct consequence of the tumor uncontrolled growth, is microenvironmental acidosis. Due to extremely variable conditions of the tumor mass perfusion, cells might be exposed to acidosis for either short or long periods. It is therefore difficult to identify a common and universal marker of acidosis [27]. Recently it was reported that Carbonic anhydrase IX (CAIX), a well-known marker of hypoxic tumor regions, is also subjected to a significant mRNA and protein expression upregulation in melanoma, breast and colorectal cancer cells exposed to transiently or chronically acidified medium (pH 6.7 ± 0.1), independently from hypoxia [28–30]. CAIX is also involved in the acquisition of chemo- and radio-resistance, and activation of the PI3K/mTOR/p70S6K signaling pathway [31]. EMT is also regulated by inflammation throughout a series of cytokines produced and secreted by stromal cells that induce EMT [32]. Among the numerous cytokines reported, the most important ones are Transforming Factor Growth beta-1 (TGF- β 1), Tumor Necrosis Factor-alpha (TNF α), Notch and Wnt. In particular, TGF- β 1 plays a critical role in EMT induction and carcinogenesis. At low concentration and at early tumor stages, TGF- β 1 has an anti-tumor effect by inducing growth arrest and apoptosis, while at higher concentrations during tumor progression, it gains a pro-tumorigenic role, by inducing EMT, migration and invasion of cancer cells [33]. TGF- β 1, both in physiological and pathological conditions is reported to be an effective EMT inducer in mammary, lung, pancreatic and other tissues by activating SNAIL (Snail) and SNAIL2 (Slug) through the canonical SMAD signaling pathway [34, 35]. TGF- β -induced EMT can also be driven by microRNA (miRNA) since the zinc-finger E-box-binding homeobox 1 (ZEB1) and

ZEB2 expression are regulated by the pro-epithelial miR200 family and miR205 (discussed in “Non-coding RNAs and EMT”), which are downregulated by TGF- β via the Akt serine/threonine kinase pathway [36]. TGF- β 1 regulation of EMT was recently described in melanoma to be mediated via upregulation of the urokinase plasminogen activator receptor, which in turn stimulates metastasis [37], as well as in pancreatic cells, through an increase of mitochondrial mass and ROS, and decrease of mitochondrial membrane potential [38].

Cadherin expression pattern in EMT

One of the typical traits of EMT is the downregulation of E-cadherin and upregulation of N-cadherin, reflecting the loss of epithelial cell polarity, a reduction in cell–cell adhesion, and the acquisition of mesenchymal traits. In particular, the loss of E-cadherin expression is associated with increased migratory and invasive phenotype, as well as the acquisition of anoikis resistance [39]. This switch, although commonly reported in many tumors, is only partially executed, such as in ovarian carcinoma and in lung adenocarcinoma [40]. E-cadherin was reported to be deregulated by hypermethylation in several cancers as well as by cis-acting factors including Snail1, Slug, Twist, ZEB1 and ZEB2 [41, 42]. E-cadherin is also downregulated by miR9 in esophageal squamous cell carcinoma demonstrating that this phenomenon could be orchestrated by many pre- and post-transcriptional pathways [43]. Therefore, to date, regulation of E-cadherin represents a major target to inhibit EMT, and this approach has been explored via both gene therapies by knocking out miRNAs targeting its suppressors, and by pharmacological approaches [44–47].

EMT related metabolic alterations

During EMT, cell metabolism is subjected to radical changes, however, the nature of these changes appears to be context- and tumor-dependent [48]. Indeed, the enhancement of both oxidative phosphorylation (OxPHOS) and glycolytic activity is suggested to be associated with EMT. Some studies reported an enhanced OxPHOS metabolism induced by peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), whose expression is associated with EMT, metastasis and poor prognosis of patients with invasive ductal carcinomas [49]. Other studies, instead, described that rapidly growing tumors often face increased hypoxic condition that induces and stabilizes HIF-1 α , a master regulator of glycolysis and a well-known EMT inducer, thus potentially

connecting glycolysis with EMT [23]. These discrepancies in metabolism could be due to the highly plastic nature of EMT, leading cancer cells to express different sets of epithelial and mesenchymal markers in the so-called hybrid states [50].

EMT associated aggressive phenotype

Although mechanisms driving EMT are relatively well described, those governing the metastatic cascade are still very much debated. The metastatic cascade consists of four main consecutive steps: local invasion of adjacent tissue of the primary tumor, intravasation, extravasation, and colonization of distant organs [51]. Due to the extreme difficulties to monitor this complex process in vivo, it is speculated that during invasion, carcinoma cells modulate their cell–cell adhesion, and acquire migratory and invasive capacities. In blood or lymphatic vessels, CTCs acquire anoikis resistance to survive shear stress and escape immune surveillance. Notably, in breast cancer, CTCs may exhibit intermediate epithelial–mesenchymal phenotype, that can function as a critical driver for tumor progression and metastasis [52, 53]. Bioinformatic analysis of CTC transcriptomes also shows that CTCs exhibit intermediate values in the EMT scoring [54, 55]. Two recent studies using spontaneous breast-to-lung metastasis mouse models [56] and mouse pancreatic ductal adenocarcinoma models [57] claimed that EMT is dispensable for metastasis but plays a major role in chemoresistance, however this has raised debate and conflicting view on the importance of EMT in metastasis. However, it is now clear that carcinoma cells engaging in the metastatic process display different degrees of EMT phenotypes [58–61]. The role of more epithelial-like carcinoma cells in metastasis could, however, be envisioned in the primary tumor at the invasive front or in the blood flow [11]. Indeed, a report has shown that EMT cancer cells can invade adjacent connective tissues, and intravasate, but they are unable to form metastasis until co-injected with non-EMT cells, that may play an important role in adhesion to the lung vasculature and extravasation. Hence, cooperation between the non-EMT and EMT cells completes the metastatic process [9]. Moreover, it should be pointed out that CTCs cannot be assumed to be CSCs. CTCs exhibit a spectrum of EMT plasticity and express some stem cell markers [62], however, only the CTCs with indefinite self-renewal ability have real CSC characteristics [63]. Another CSCs typical feature is the acquisition of drug resistance due to unbalanced anti-apoptotic protein expression, increased levels of ATP-binding cassette (ABC) transporters and the non-proliferative status of stem cells [10].

EMT-related genes and signaling pathways

Several signaling pathways are involved in EMT including TGF- β , fibroblast growth factor (FGF), hepatocyte growth factor (HGF), Wnt, Notch, and Hedgehog (Fig. 1) [64]. Snail1/2, Twist and ZEB1/2 are common downstream transcriptional factors (TFs) of these pathways. However, these TFs act differently to initiate EMT [65]. Modulation of expression of multiple markers is observed during the progression of EMT. Epithelial genes, such as zonula

occludens-1 (ZO-1), occludin and E-cadherin, are down-regulated whereas mesenchymal genes, such as Fibronectin, Vimentin and N-cadherin, are elevated [66]. Snail1/2 and ZEB1/2 directly bind to E-cadherin to suppress its activity, while Twist indirectly represses E-cadherin [42, 67].

EMT is involved in the development, fibrosis, wound-healing and carcinoma metastasis [68–70]. During early embryonic development in vertebrates, epithelial cells of the epiblast undergo transient EMT during gastrulation to form mesoderm and definitive endoderm; similarly, neural

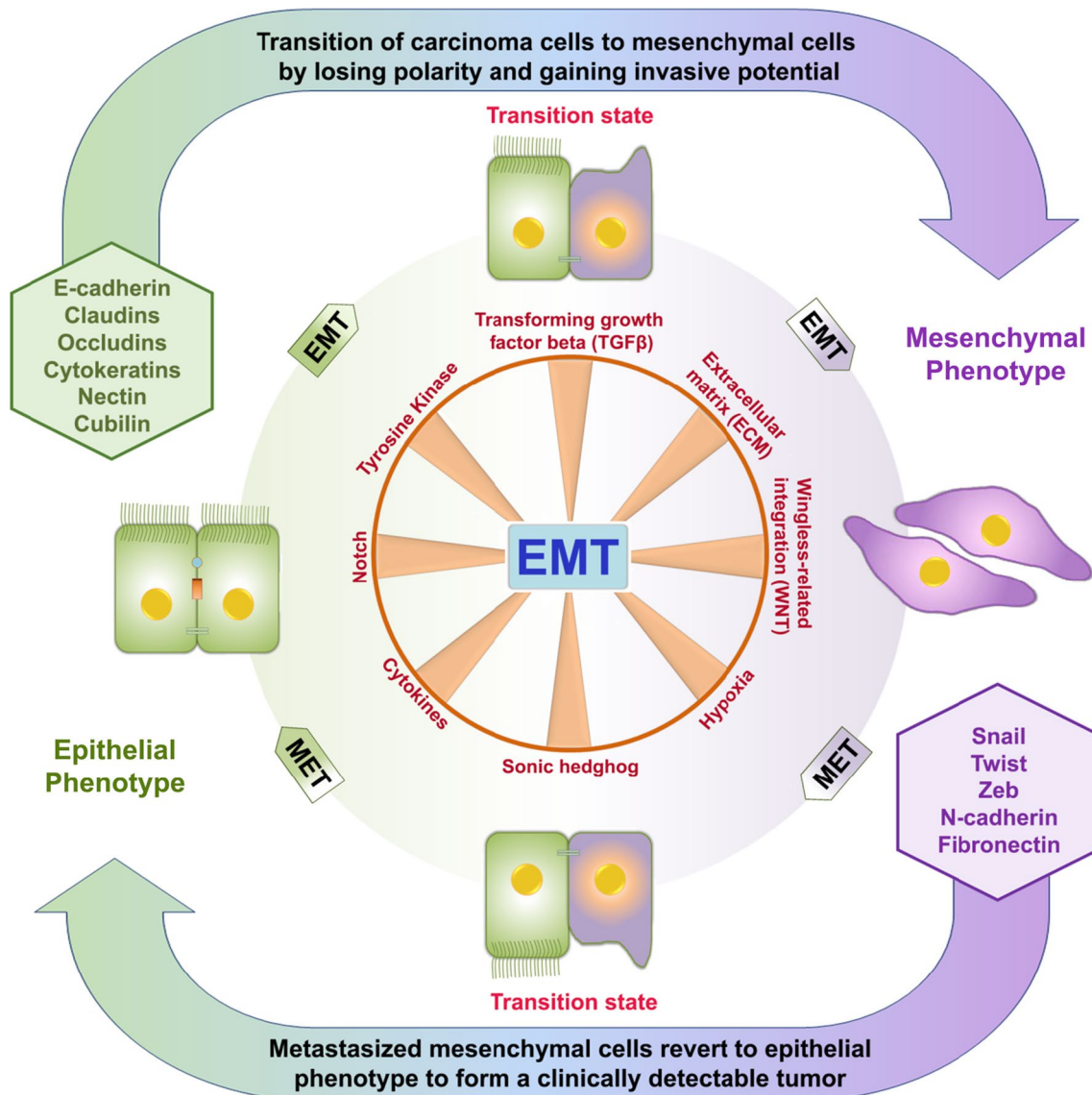


Fig. 1 Distinct intermediate states of epithelial–mesenchymal transition in the progression of carcinoma. EMT is a functional transition of differentiated epithelial cells into migratory mesenchymal cells. A list of EMT-inducers and common markers expressed in the epithelial and mesenchymal cells during EMT and its reversal process MET are provided. Colocalization and combinatorial expression of these

distinct markers define intermediate EMT phenotypes. EMT enables the transitioned mesenchymal-like carcinoma cells to reduce or lose intracellular adhesion and gain invasive potential. This allows them to intravasate into the blood and lymph vessels and subsequently extravasate to form micrometastases, which can revert to an epithelial-like state through MET and thus forming a clinically detectable tumor

crest cells dissociate from the dorsal border of the neural epithelium by EMT before migrating in different territories of the embryo [71]. In the embryonic heart, Notch signaling promotes EMT by transcriptional induction of Snail, which leads to the development of cardiac valve [72].

TGF- β signaling has been shown to activate fibrosis and wound-healing [73]. Downstream signaling of the TGF- β pathway, Smad2/3, plays a key role in activating Snail, ZEB and Twist expression [74]. Studies showed that elevation of TGF- β -Smad signaling pathway induces EMT marker production causing fibrosis in many tissues/organs such as lens [75], kidney [76–78], liver [79], and lungs [80]. Indeed, surgical replacement of cataractous lens induces TGF- β secretion in capsule, facilitating induction of wound healing in lens capsular epithelial cells. However, excess of TGF- β in capsule leads to abnormal growth in lens epithelial cells (LECs) [81], further causing posterior capsular opacification (PCO), also referred to as secondary cataract. Recent studies indicated that aldose reductase (AR) [82, 83] and α B-crystallin [84] are involved in TGF- β 2-induced EMT in LECs. A blockade of AR or α B-crystallin might delay the onset and/or progression of development of PCO.

Other than TGF- β -Smad signaling, FGF and its downstream pathways, RAS/MAP kinase and PI3/AKT pathways, are another EMT inducing signaling serving many functions in both the developing and adult organisms [85]. However, FGF shows an opposing effect against TGF- β -Smad signaling by inducing suppressive micro-RNA-20a [86]. FGF signaling also plays essential roles in embryonic development by downregulating E-cadherin [87], and in vertebrate development by activating phosphorylation of Smad1 [88].

In cancer cells, Wnt activation suppresses the destruction complex of glycogen synthase kinase 3 beta (GSK-3 β), facilitating β -catenin translocation into the nucleus. This process induces the transcriptional production of Snail, leading to downregulation of E-cadherin [89, 90].

Jagged/Notch signaling regulates differentiation, metabolism and angiogenesis of cancer cells [91]. After activation, Notch intracellular domain (NICD) is released via a cascade of proteolytic cleavage and translocated into the nucleus, leading to transcriptional expression of Snail1/2 and Twist [90]. ZEB1/2 are another transcriptional suppressors of E-cadherin, which are found in carcinogenesis of diverse cancer types including melanoma [92], gastric cancer [89] and breast cancer [93]. In addition, Notch signaling is induced by TGF- β signaling to initiate EMT in epithelial cells from mammary glands, kidney tubules, epidermis [94], and embryonic retina [95]. In non-small cell lung cancer (NSCLC) carcinogenesis, Notch signaling is involved in EMT-triggered metastasis [96].

To date, therapeutic resistance is one of the most challenging issues in cancer therapy. Therapeutic resistance mainly includes chemoresistance, endocrine resistance,

targeted drug resistance and radiation resistance [97]. In the aspect of chemoresistance, chemokines are known to promote cancer cell migration and metastasis [98, 99] and EMT plays an important role in this process. For instance, interleukin 6 (IL-6) is able to induce Snail expression through STAT3 signaling [100]. In addition, TNF- α , a crucial cytokine involved in cancer progression renders breast cancer resistance through NF- κ B-induced EMT activation, resulting in loss of E-cadherin and gain of Twist, Snail and ZEB [101]. A recent study showed that ubiquitin-specific protease 1 (USP1) stabilizes Snail via de-ubiquitination, leading to ovarian cancer cell resistance to platinum [102]. EMT has been increasingly recognized to have a critical role in cancer drug resistance. Indeed, targeting EMT to overcome drug resistance has been the subject of some clinical trials [90, 103].

Non-coding RNAs and EMT

The existence of non-coding RNAs (ncRNAs) was not demonstrated until the discovery of small nuclear RNAs (snRNAs), in 1986, as a possible player in the excision of introns from nascent mRNAs and later small nucleolar RNAs (snoRNAs) joined the clamour [104]. The advent of sensitive high-throughput genomic technologies provided an unprecedented ability to detect the vast majority of novel transcripts, which surprisingly, seem not to be derived from annotated protein-coding genes. Indeed, while ~75% of the human genome is transcribed to RNA, only ~1.2% is responsible for encoding proteins [105]. Thus, a large portion of the genome is transcribed into non-coding RNAs, which are thought to be dedicated to *cis* and *trans* regulatory functions.

In the early 2000s, the non-coding RNA biology gained attention with the discovery of miRNAs, ~22 nucleotide non-coding regulatory RNAs, emphasizing the prominence of post-transcriptional events in gene expression in eukaryotic organisms. There are more than 2000 miRNAs that have been annotated in the human genome, several of which have since been identified as a putative component of oncogenic and tumor suppressor networks. It is obvious that loss- or gain-of-function of specific miRNAs aids in cellular transformation and tumorigenesis since the miRNA expression patterns are highly specific for cell-type and cellular differentiation status. Over 90% of cancer-related deaths are due to the metastatic events that are initiated by deregulation of EMT regulatory miRNAs. Deregulation of the miR200 family, miR200a, miR200b, miR200c, miR141 and miR429, is one of the major events in EMT [106]. Expression of the miR200 family is downregulated at the transcriptional level by ZEB1 and Snail factors, providing a positive regulatory loop to maintain the expression of ZEB1 [107], an essential

gene in the initiation of EMT [108, 109]. Recent studies have highlighted a prime EMT inducing role of miR103/107, which modulates multidrug resistance in human gastric carcinoma and metastasis of oral, breast and colorectal cancer [110–112]. miR21 expression was elevated in several human tumors and related to the development of therapy resistance through EMT in p53 mutant tumors [113]. In oral cancer, let7d functions were reported as novel regulators of EMT that promote the regulation of chemoresistance [114]. Likewise, altered expression of miR21, miR125b-2, miR134, miR155, miR184, and miR205 was reported in aggressive oral squamous cancers in which EMT is the major driver event for metastasis and chemoresistance [108, 115, 116].

Nevertheless, several miRNAs act as tumor suppressors which could serve as an early biomarker in EMT-associated cancer progression. For example, miR328, miR876-5p, miR30a, miR302b/c/d, miR199b-5p, miR148, miR335 and miR140 inhibit EMT in several human cancers through modulating the mesenchymal marker genes such as TWIST1, BMI, ZEB1/2, Vimentin and TGF- β expression, and sensitize the tumors to chemo or radiotherapy [117–119]. Apart from the downregulation of tumor suppressor miRNAs, overexpression of miR197 in lung, miR708 in melanoma, miR224 in bladder, miR216a in ovarian and miR92a in lung cancers through modulating PTEN/PI3K/AKT signaling accounts for enhanced proliferation, invasion, metastasis to local or distal regions and promotes EMT [97, 120–123]. Taken together, several functional studies demonstrate that deregulated miRNAs exert a promoting role in EMT-related cancers and can serve as promising biomarkers and attractive therapeutic targets.

In the midst of these newly discovered RNA elements, long non-coding RNAs (lncRNAs), greater than 200 nucleotides in length, are thought to have functional roles in an array of cellular functions such as development, differentiation, proliferation and cell fate determination, in addition to pathogenesis [124]. Although thousands of lncRNAs have been identified, most of their functions are yet to be revealed. Here, we focus on some of the well-studied lncRNAs that have a significant role in EMT and cancer therapy resistance. lncRNA XIST in non-small cell lung carcinoma and lncRNA ZEB2-AS1 in oral squamous cell carcinoma promotes metastasis through the deregulation of TGF- β , WNT/ β -catenin and ZEB pathways [108]. CCAT1 and CCAT2 that are found to be overexpressed in several cancers, were super-enhanced by the oncogene MYC and contributes to chemoresistance via sponging several tumor-suppressive miRNAs [125, 126]. NORAD, a cytoplasmic lncRNA, regulates TGF- β signaling and EMT-like phenotype in lung adenocarcinoma and pancreatic cancer [127]. Overexpression of lncRNA CRNDE facilitates EMT phenotype in intrahepatic cholangiocarcinoma, bladder cancer, thyroid carcinoma and modulates PI3K/AKT signal

and Notch1 pathways to enhance EMT in gastric cancer and osteosarcoma, respectively [125, 126, 128]. Cancer stemness is principally responsible for EMT related tumor reoccurrence and therapeutic resistance and is promoted by several of the lncRNAs such as ROR, OIP5-AS1, SOX2OT, NEAT1, HOTAIR and DANCER [108, 125, 129].

Many studies have established the role of lncRNAs in a competitive regulatory interaction, known as competing endogenous RNA (ceRNA) networks, wherein lncRNAs can act as miRNA decoys to modulate gene expression. Such interactions are often interconnected, hence the aberrant expression of either miRNA or lncRNA could disrupt the complex regulatory network leading to disease pathology [130]. H19, TUG1 and MALAT1 co-operatively sponge miR21 and miR148a and promote tumor by overexpressing downstream targets EGFR, KLF4, DNMT1 and AGO4 in gastric cancer [108]. Similarly, CCAT1, ROR, ZEB2-AS1 and OIP5-AS1 sponge several anti-proliferative and metastatic inhibitory miRNAs in oral cancer and account for EMT progression and poor therapeutic outcomes [108, 125]. XIST accounts for EMT in non-small cell lung carcinoma by activating TGF- β pathway through sponging miR367/141 [131]. Likewise, overexpressed CRNDE and DANCER promote cell proliferation, invasion and metastasis by competing to bind with the miR384 in papillary thyroid cancer and miR33a-5p in osteosarcoma, respectively [125, 129]. In addition, adding another layer of complexity to the ceRNA regulatory network, miR203 was found to inhibit HOTAIR in renal cell carcinoma and regulates tumorigenesis through EMT [132]. Similarly, miR192 and miR204 were also identified to target lncRNA HOTTIP which targets GLS1 as its downstream target to promote tumor progression in hepatocellular carcinoma [133].

CRISPR/Cas systems

Studying and manipulating the genetic mechanisms underpinning cancer biology and pathogenesis is critical and demands a comprehensive genetic toolkit for efficient, precise, and scalable genetic manipulation. Such functional genomic technologies are crucial to our ability to study and modify cancer cells and cellular processes involved in cancer progression. New advances in genomic biotechnologies—particularly CRISPR/Cas-based tools—have revolutionized our ability to perform targeted genetic manipulations in diverse organisms and been instrumental in enabling genome editing across a diversity of cell types and model systems [134].

CRISPR, or clustered regularly interspaced short palindromic repeats, is a group of DNA sequences in prokaryotes that play an important role in bacterial and archaeal immunity, and have been co-opted for genome editing across the

tree of life [135, 136]. In type II CRISPR systems, so-called “spacer” sequences from CRISPR loci (which are derived from viral, plasmid or other foreign DNA that have been inserted into the CRISPR locus) are transcribed into RNA, and processed into small CRISPR RNAs (crRNAs), which then associate with CRISPR-associated (Cas) proteins and a trans-activating crRNA (tracrRNA). Together, this complex is targeted to “protospacer” sequences based on base-pairing complementarity between the protospacer and the crRNA sequence, and the presence of a protospacer adjacent motif (PAM) site. Cas proteins have endonuclease ability and can cause double-strand breaks (DSBs) at the target protospacer locus.

While this system is natively used by prokaryotic cells to detect and cleave invading foreign DNA, more recently, it has also been exploited as a powerful biotechnology tool for precise genome editing at a targeted genetic locus in mammalian cell lines [134]. This precision editing of CRISPR was first demonstrated using the Cas9 protein from *Streptococcus pyogenes* and a modified chimeric single small guide RNA (sgRNA), which was a fusion of both the crRNA and tracrRNA. By manipulating the sequence of this sgRNA, the Cas9 endonuclease could be programmed to target a specific DNA sequence and generate a DSB [135]. These DSBs are then repaired by cellular processes and machinery that protect the cell from DNA damage. In general, these DSBs can be repaired in one of two ways: by non-homologous end joining (NHEJ), which is mutagenic, and can result in insertions or deletions (indels) at the target locus, or by homology-directed repair (HDR). The latter repair strategy can only be exploited by the cell if a DNA template with homology to the target locus is present.

Therefore, by expressing these components in a desired cell line (the Cas9 endonuclease, a sgRNA targeting a region of interest, and a DNA template to be used for HDR), researchers are able to make precise DSBs, and can direct HDR using a supplied template to generate specific mutations or alterations at the target locus. Since the first example of CRISPR based genome editing [135], a number of other studies quickly demonstrated the power of CRISPR-based gene editing for in vivo applications [137–140]. Since then, several new variants of these CRISPR technologies have been developed. Several of these technologies take advantage of a nuclease-dead version of the Cas9 enzyme (dCas9), which can no longer generate DSBs, but retains the ability to be guided by sgRNA to targeted genomic loci. For instance, CRISPR interference (CRISPRi) exploits dCas9 fused to the transcriptional repressor, along with sgRNAs targeting promoter regions in order to silence transcription and knockdown expression from specific genes [141, 142]. CRISPR-based gene activation or overexpression (CRISPRa) [141, 143], uses a similar strategy and functions by fusing the dCas9 protein to transcriptional activator proteins.

Fusing dCas9 with other enzymes, including those involved in epigenetic modifications, can be used to target specific genomic regions with targeted epigenetic marks [144–146]. Recent additions to the CRISPR technology toolkit include CRISPR-based precise DNA base editing technology [147], and CRISPR-based RNA editing [148].

Together, all of these CRISPR technologies are powerful tools for precise genome editing and regulation and have been extensively applied to cancer research [149]. CRISPR-based genome editing has revolutionized the field of gene therapy, which holds immense potential for applications in cancer therapeutics [150]. If CRISPR editing can be effectively used in vivo to genetically target and modify cancer-related mutations, tumor suppressor genes, or oncogenes, then this would be a powerful gene therapy against cancer progression. As cancer cells notoriously accumulate many genetic mutations, CRISPR/Cas editing might be applied to target and correct such genetic mutations as an anticancer strategy. Further, since epigenetic modifications are known to be critical for cancer cell development, using dCas9-based tools for targeting epigenetic regulation may also prove an important therapeutic tool against cancer progression.

CRISPR/Cas9-mediated EMT gene editing

As discussed, EMT is one of the major mechanisms that contribute to the development of an organism. On the contrary, EMT has also been shown to play a key role in the progression of various pathologies such as cancer and fibrosis. A number of techniques have been employed in order to ascertain the role of diverse genes in the regulation of EMT during various disease conditions. RNA interference or RNAi is one such powerful molecular tool that has been used to study the role of genes involved during EMT in vitro. However, knockdown of target gene expression by RNAi in vivo is impractical due to its non-specificity to the target cell type, as well as its temporary nature. The recent advances in molecular techniques involving RNA-guided Cas9 nuclease from the microbial CRISPR adaptive immune system are being used to facilitate the efficient genome engineering in eukaryotic cells and overcome the pitfalls of the RNAi technique. The CRISPR/Cas9 gene-editing technique is now being used to investigate its efficiency in editing genes in various disease conditions such as cancer, cataract and fibrosis. The CRISPR/Cas9, editing genes at the DNA level, helps us to identify novel EMT-related genes [151]. These genes could be used as a potential therapeutic target in EMT-related diseases [151] and this technique applied as an emerging tool for precision therapy (Fig. 2) [152].

This is exemplified by effective gene editing using CRISPR/Cas9 system, achieved for tropomyosin (Tpm) in the lens epithelial cells (LECs). Normal rat LECs,

Potential applications of CRISPR/Cas systems for EMT study

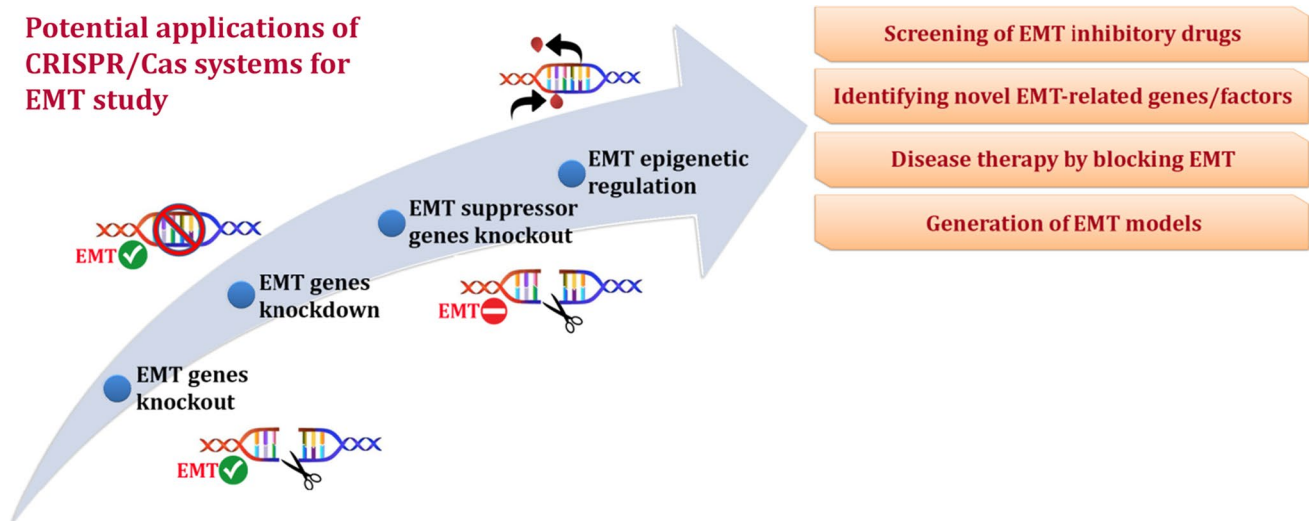


Fig. 2 CRISPR toolkit is applied to study EMT. Different approaches including gene knockout, knockdown, knock-in, and epigenetic regulation are achieved by the CRISPR/Cas technology to study EMT-related genes and pathways and identify novel drugs to control the EMT process

express low levels of tropomyosin (Tpm) isoforms, Tpm1 and Tpm2. However, these isoforms were shown to be increased during EMT, correlating with fibrosis observed in the rat PCO model. In order to understand the role of Tpm in injury-induced EMT in the lens, CRISPR/Cas9 gene editing was used to knockout *tpm* gene which ultimately led to preventing EMT, and thus lens opacity and cataract [153].

Genes implicated in cancer EMT were also edited by CRISPR/Cas9. One study specifically abrogated Snail1 expression in human ovarian adenocarcinoma (RMG-1) cells and showed that Snail1 is critical for TGF- β -induced EMT in RMG-1 cells [154]. Fibronectin (FN), one of the major extracellular matrix proteins required for cell adhesion, is a well-known EMT marker. An alternatively-spliced fibronectin containing the extra domain-A (EDA), is expressed at the sites of migrations in embryos, wound-healing and cancers, while EDA is excluded in normal adult tissues. The peptide motif (CTYSSPEDGIHEC) within EDA is necessary for the binding of integrin $\alpha 9 \beta 1$, which remodels actin cytoskeleton to facilitate cell adhesion, proliferation and EMT in cancer cells. EDA positive fibronectin (EDA + FN) is shown to be increased in response to pro-tumorigenic factors such as TGF- β and TNF α in highly aggressive salivary adenoid cystic carcinoma (SACC) [155]. Several approaches have been applied including siRNA to knockdown EDA + FN, however, these approaches have been unsuccessful. For example, knockdown of EDA + FN by siRNA actually decreases the total amount of FN [155], while FN is vital to normal tissues. CRISPR/Cas9 EDA editing, allowed to abrogate the pro-oncogenic effects of EDA + FN and to induce inhibition of tumor progression both in vitro and in vivo [129].

Cancer cell survival depends on the ability of cells to evade apoptosis that requires the activity of an array of proteins known as inhibitor of apoptosis (IAP). One of the major IAP is survivin, which is highly expressed in various human cancers while its expression is low in fully differentiated normal adult tissues. Survivin expression is known to be associated with tumor metastasis and chemoresistance in various cancers such as melanoma, renal, prostate, and breast. In a recent study CRISPR/Cas9-mediated editing of survivin gene (BIRC5) in ovarian cancer cell types, SKOV3 and OVCAR3, led to the inhibition of cell proliferation, migration, and invasion, and sensitized cell response to chemotherapy. This study also revealed that survivin may contribute to tumor metastasis and chemoresistance by promoting EMT through the TGF- β pathway in ovarian cancer cells [156]. A single-cell CRISPR–Cas9 screen was recently used to identify the genetic machinery that controls spontaneous- or TGF- β -mediated EMT [157]. This study identified targets of KRAS to be critical throughout the EMT continuum [157]. Recently, Qu et al. showed that c-Myb transcription factor promotes colorectal cancer (CRC) carcinoma through c-fos-induced EMT. c-Myb KO established by CRISPR/Cas9 inhibited the xenograft growth and metastasis, and induced tumor apoptosis [158]. Furthermore, it was shown that Snail1 employed β -Catenin-LEF1 complexes to redirect Wnt/ β -Catenin pathway activity [159]. Meanwhile, applying CRISPR/Cas9 technique, identified beta-lactamase-like (LACTB) as a novel epigenetic silenced tumor suppressor. It modulates the stability of p53 in CRC [160]. Tn antigen exposure mediating Core 1 $\beta 3$ Gal-T specific molecular chaperone (Cosmc) knockout using CRISPR/Cas9 promotes H-Ras-induced EMT activation and CRC metastasis [161]. A recent study showed that CRISPR/Cas

knockout of fyn-related kinase (FRK) in H1299 lung cancer cells markedly reduced cell invasion and EMT [151]. Table 1 includes studies where CRISPR/Cas technology has been utilized to study EMT by targeting EMT-related genes.

Most of the studies involving the use of CRISPR/Cas9 system have been conducted in vitro; however, the effective use of this novel gene-editing technique in vivo is under investigation. The challenge is finding safe delivery methods to target organs efficiently [162, 163]. Recent advances in delivery systems including viral or non-viral vectors emerged the in vivo application of CRISPR/Cas gene-editing technique [164–167]. For example, Jubair et al. reported systemic delivery of CRISPR/Cas9 to knock out oncogenes and eliminate tumors effectively [162]. Genome editing has broad utility in animal models, such as the establishment a novel GADD45G mutated rabbit model to investigate the link between EMT and lip development. Results showed GADD45G^{-/-} rabbit embryos show significant overexpression of E-cadherin protein in the epithelium and decrease in Vimentin protein level within the mesenchyme compared to wild-type [168].

CRISPR/Cas9-mediated knockout of EMT associated non-coding RNAs

Identifying the function of a non-coding transcript is one of the most central parts in understanding the EMT progression. Screening differentially expressed ncRNA's function in a cancer cell by RNAi-based gene knockdown libraries (loss-of-function) and overexpression libraries as a transgene (gain-of-function) is tedious and time-intensive. In addition, RNAi has a potential limitation of incomplete silencing or off-targeting, especially, miRNA-like off-target repression when homologous sequences encountered [169]. Even though shRNA/siRNA or anti-sense oligonucleotides present some advantages, the main obstacles remain the cost and the size of the libraries, and the efficiency of delivery to the target site/tissue; In addition they are short-term treatment strategies [170–172]. More recently, the CRISPR/Cas system has been established as an efficient genome editing tool, highlighting its application, using sgRNA to target dysregulated non-coding RNA genes [138]. Unlike targeting the transcript for degradation, the CRISPR/Cas system brings the solution for long-lasting treatment or management and hence can be used for the systematic identification of functions of non-coding transcripts in EMT [173].

However, the main concern for using this method at present is the off-target effects because most of the lncRNAs are transcribed from anti-sense or from within a part of a coding gene, which means they share their genomic segments with another coding gene [174, 175]. Similarly, often miRNAs are encoded within introns of genes, making

it particularly challenging to achieve robust CRISPR/Cas-based editing. In addition, the regulation of gene expression and genome architecture differs between mouse and human, which makes it complex to extrapolate the effect in human cells [176]. However, RNA editing by using catalytically-inactive Cas13 (dCas13) and LwaCas13a aided RNA interference provides a flexible platform for studying RNA editing and/or targeting in mammalian cells [148]. The CRISPR libraries designed for ncRNA loss-of-function or gain-of-function help in identification of the role of non-coding transcripts in cancer. Furthermore, these libraries permit access to the longer non-coding transcripts that are not available in cDNA libraries [177]. Recently, a gene segment was deleted from lncRNA CCAT1 using CRISPR du-HITI (dual allele homology-independent targeted integration system) and inserted a reporter and a transcription termination signal to generate CCAT1 knock-out cell line [178], which can be employed to edit or delete any lncRNA gene to study its role in EMT.

On the other hand, repression of miRNA expression by targeting the terminal loop and by using specifically designed gRNAs that direct Cas9 to cut at a single site of a miRNA gene resulting in knockdown of the miRNA was successfully achieved [179, 180]. miRNA gene silencing was achieved using CRISPR/Cas9 by transfecting vectors carrying sgRNAs specifically designed to target miR17, miR200c, and miR141 genes, well known EMT regulatory miRNAs, in colon cancer cell lines [181]. miR21 is one of the first mammalian miRNA identified that is strongly conserved throughout evolution. A number of targets of miR21 such as Bcl2 [182], PTEN [183], RhoB [184], TGFBR1 [185] and tropomyosin [186], have been experimentally validated. In order to demonstrate the role of miR21 during EMT, miR21 expression was disrupted by transducing SKOV3 and OVCAR3 ovarian cancer cells with four different miR21 gRNA lentiviral CRISPR/Cas9 vectors that target different regions of the pre-miRNA sequence [187]. This study linked the loss of miR21 to inhibition of EMT, cell proliferation, migration, invasion and sensitization of cells to chemotherapy. CRISPR/Cas9 was used to target hypoxia-inducible miRNAs, miR210-3p and miR210-5p, which are processed from pre-miRNA210, in renal cell carcinoma cell lines [188]. More recently, a cell type-specific Cas-ON switch based on miRNA-regulated expression of anti-CRISPR proteins (Acr) was developed [189]. Using this system, Cas9 activity was only released in hepatocytes or cardiomyocytes but was repressed in off-target cells [189]. In particular, using cell-type-specific CRISPR/Cas-ON systems functions of EMT specific non-coding gene can be effectively retrieved by turning on or off based on cell polarity. In addition, developing an inducible Cas-ON system to tweak a specific EMT regulatory non-coding RNA gene in transiting tumor cells is another strategy to study EMT.

Table 1 EMT genes edited by CRISPR/Cas9

Target genes	Disease	Models	Methods	Major outcomes	References
Tropomyosin 2 (Tpm2)	Posterior capsule opacification (PCO)	Lens epithelial cells	KO	Inhibition of injury-induced EMT in PCO	[153]
Mothers against decapentaplegic homolog 3 (SMAD3)	Fibrosis	Normal human lung fibroblasts (NHLFs) and human bronchial epithelial cells (HBECS)	KO	Inhibition of fibroblast to myofibroblast transition (FMT) and EMT via decrease in alpha smooth muscle actin and fibronectin 1	[190]
HECT domain E3 ubiquitin protein ligase 1 (HECTD1)	Silicosis	Mouse endothelial cell line (MML1)	Activation	Increased expression of mesenchymal markers and downregulation of epithelial marker genes	[191]
Zinc finger CCH-type containing 4 protein (ZC3H4)	Silicosis	MLE-12	KO	Blockage of EMT and cell migration induced by silicon dioxide	[192]
Protein kinase C α (PKC α)	Ovarian cancer	OCSC1-F2 cells	KO	Regulation of Twist-1 protein expression	[193]
Integrin α 3	Breast cancer	MDA-MB-231 cells	KO	Suppression of the sialylation of membrane proteins, such as the EGFR, and reduction in cell migration and adhesion	[194]
CXC chemokine receptor 4 (CXCR4)	Hepatocellular carcinoma	HepG2 cells in vitro and in mice xenograft	KD	Reverse of EMT; reduction in cell proliferation, migration and invasion; increase in chemosensitivity	[195]
Nuclear receptor coactivator 5 (NCOA5)	Hepatocellular carcinoma	LM3 cells	KO	Suppression of EMT, leading to impaired cell proliferation and migration	[196]
Ovo like zinc finger 2 (OVOL2)	Nasopharyngeal carcinoma	CNE2 and HNE1 cells in vitro and in xenografts	KO	Promotion of EMT, cancer stemness and metastasis	[197]
Extra domain-A (EDA) exon	Nasopharyngeal and colorectal cancers	CNE-ZZ and SW480 cells	KO	Inhibition of proliferation, migration, invasion and EMT	[129]
Zinc finger E-Box binding homeobox 1 (ZEB1)	Colorectal cancer	LS174T	KO	ZEB1 is neither required nor sufficient for Snail1-induced EMT	[198]
C1GALT1 encoding T-synthase	Colorectal cancer	HCT116 cells	KO	Aberrant O-glycosylation, promotion of cell proliferation, adhesion, migration and invasion via induction of EMT	[199]
Sterile α motif- and Src-homology 3-domain containing 1 (SASH1)	Colorectal cancer	HCT116 cells in vitro and in an orthotopic mouse model	KO	Promotion of EMT, cell invasion and chemoresistance	[200]
Cytoskeleton regulator RNA (lncRNA CYTOR)	Colorectal cancer	RKO, SW480, SW620, HCT116, HCT8 and in vivo xenograft	KO	Activation of NF- κ B pathway by forming heterotrimeric complex with NCL and Sam68 and induction of EMT	[201]

Table 1 (continued)

Target genes	Disease	Models	Methods	Major outcomes	References
Caspase-3 (CASP3)	Colon and breast cancer	HCT116 and MDA-MB-231 cells	KO	Reduced EMT phenotypes and pulmonary metastasis by increasing E-cadherin, and reducing N-cadherin, Snail, Slug and ZEB1 expression, as well as reduction in cell migration and invasion	[202]
Tumor protein 53 (TP53)	Colon cancer	COLO 320DM, WiDr, and SW48	Knock-in mutation	Promotion of induced pluripotent stem cells and EMT	[203]
miR210-3p	Renal cell carcinoma	786-o, A498 and Caki2, and in vivo xenograft	KD	Promotion of EMT and cell growth by TWIST1 up-regulation	[188]
Von Hippel Lindau (VHL)	Renal cell carcinoma	RENCA	KO	Promotion of HIF-1 α -mediated EMT, tumor aggressiveness and metastasis	[204]
Phosphoinositide-3-Kinase Regulatory Subunit 1 (PIK3R1)	Renal cancer	786-O and A-704 cells	Haploid KO	Promotion of proliferation, migration and EMT through the AKT/GSK3 β /CTNNB1 pathway	[205]
Fibronectin EDA exon	Salivary adenoid cystic carcinoma	SACC-83 cells	KO	Inhibition of motility, invasion and EMT	[155]
Annexin A1 (ANXA1)	Pancreatic cancer	MIA PaCa-2 cells	KO	Induction of EMT via downregulation of miR196a	[206]
ANXA1	Pancreatic cancer	MIA PaCa-2 cells	KO	Decreased extracellular vesicles enriched in exosomes, reduced metastatic potential and inhibition of EMT	[207]
Mitogen-activated protein kinase kinase kinase 3 (MEKK3)	Pancreatic cancer	Panc1 and AsPC1 cells	KO	Reduction in EMT and cell migration, and colony formation ability	[208]
BIRC5 gene encoding survivin	Proliferative vitreo retinopathy (PVR)	Retinal pigment epithelial ARPE-19 cells	KD	Reduction of EMT and TGF- β -induced cell proliferation and migration	[209]
Mouse double minute 2 (MDM2)	PVR	ARPE-19 human retinal pigment epithelial cells	KO	Suppression of TGF- β 2-induced EMT	[210]
Cytoplasmic polyadenylation element-binding protein 2 (CPEB2)	Breast cancer	MCF10A cells	KO	Increase in cell proliferation, migration, invasion, EMT (decreased E-Cadherin, increased Vimentin, N-Cadherin, SNAIL, and ZEB1) and increased tumor-sphere formation	[211]
Podocalyxin like (PODXL)	Breast cancer	NAMEC8R and MDA-MB-231 cells	KO	Inhibition Of Extravasation Via Disruption Of Direct Interaction Of PODXL With Cytoskeletal Protein ezrin	[212]

Table 1 (continued)

Target genes	Disease	Models	Methods	Major outcomes	References
Beclin 1 (BECN1)	Breast cancer	MDA-MB-231 cells	KO	Suppression of proliferation (via inducing cell cycle arrest), migration and invasion by partially attenuating EMT signals	[213]
MacroH2A1	Breast cancer	Human mammary epithelial (HMLE) cells	KO	Isoform-specific function of macroH2A1 in regulating EMT induction	[214]
Succinate dehydrogenase (SDH)	Breast cancer	MCF10A	KD	Promotion of EMT and wound healing	[215]
Sonic hedgehog signaling molecule (SHH)	Breast cancer	MDA-MB-231 and MCF-7	KO	Suppression of Hedgehog pathway, cell migration and neoplastic invasion, alteration of expression of EMT markers	[216]
Traf2 and Nck interacting serine protein kinase (TNIK)	Breast cancer	MDA-MB-231	KO	Attenuation of Wnt/ β -catenin signaling and EMT	[217]
Mothers against decapentaplegic homolog (SMAD1/5) and activin A (ACVR1)/bone morphogenetic protein receptor type 1A receptor type 1 (BMPRI1A)	Breast cancer	NMuMG mouse mammary epithelial cells	KO	TGF- β -induced SMAD1/5 phosphorylation to induce EMT requires members of two classes of type I receptor, TGFBR1 and ACVR1	[218]
Kindlin-2	Breast cancer	MDA-MB-231 and murine 4T1 cell lines in vitro, and in mice xenograft	KO	Suppression of EMT, migration and invasion in vitro and tumor metastasis in vivo	[219]
Quaking homolog (QKI) and RNA binding fox-1 homolog 1 (RBFOX1)	Breast cancer	HMLE human mammary epithelial cells	KO	Suppression of CD44-high cell induction, expression of mesenchymal markers, and formation of mammospheres in a Snail overexpression-induced EMT model	[196]
ZEB1	Triple-negative breast cancer	Hs578T cells	KO	Removing ZEB1 was not sufficient for reverting EMT but reduced tumorigenic potential manifested by reduced migration and invasion	[220]
Snail homolog 1 (Snail1)	Triple-negative breast cancer	Hs578T cells	KO	Inactivation of Snail1 was not sufficient to completely revert EMT, but reduced cell migratory ability	[221]
Crk adaptor protein	Breast cancer	4T1 murine adenocarcinoma cell line	KO	Reduction in tumor growth and metastasis; suppression of EMT	[222]
poly(ADP-ribose) polymerase family member 3 (PARP3)	Triple-negative breast cancer	MDA-MB-231 and MDA-MB-436	KO	Suppression of growth, survival and in vivo tumorigenicity	[223]

Table 1 (continued)

Target genes	Disease	Models	Methods	Major outcomes	References
β -Catenin and γ -catenin	N/A	Madin-Darby Canine Kidney (MDCK) cells	KO	Neither β -catenin nor γ -catenin is necessary for the induction of EMT by lymphoid-enhancer-binding factor 1 (LEF-1)	[224]
SNAIL1	Ovarian cancer	RMG1 cells	KO KO	Increase in cell–cell adhesion, reduction of cell–substrate adhesion and migration, and changes in the actin cytoskeleton, inhibition of TGF- β -induced EMT	[154]
Baculoviral IAP repeat containing 5 (BIRC5), encoding survivin	Ovarian cancer	SKOV3 and OVCAR3 cells	KO	Increase in sensitivity to paclitaxel and suppression of EMT via regulation of cytokerin 7, Snail2, β -catenin, Vimentin, and TGF- β signaling	[156]
miR21	Ovarian cancer	SKOV3 and OVCAR3 cells	Disrupted miRNA expression	Reduced EMT, cell proliferation, migration and invasion via introducing mutations in the precursor miR21 sequences	[187]
Melanoma cell adhesion molecule (CD146)	Ovarian cancer	Mouse embryonic fibroblasts (MEFs)	KO	Inversion of E-cadherin and N-cadherin expression in TGF- β 1 or BMP4-induced EMT models	[225]
al-4 Galactosyltransferase (A4GALT)	Ovarian cancer	BG1 and SKOV3 cells	KO	Induction of EMT, chemoresistance and stem-like properties	[226]
E-cadherin	Ovarian cancer	RMG-1 cells	KO	Loss of the cell adhesion, increase in cell–extracellular matrix (ECM) interactions and decrease in the cell migration	[227]
Metal regulatory transcription factor 1 (MTF1)	Ovarian cancer	SKOV3 and OVCAR3 cells	KO	Reduced cell proliferation, migration, invasion and inhibition of EMT	[228]
KH RNA binding domain containing, signal transduction associated 1 (P62 or KHDRBS1)	Prostate cancer	DU145 cells stably overexpressing P62	KO	Suppression of cell proliferation, migration and invasion, and reversal of EMT	[229]
TGF- β receptor type II (TGFBRII)	Prostate cancer	DU145 cells in vitro, and in vivo xenografts	Dominant-negative mutation	Disruption of TGF- β -induced EMT; attenuation of cell migration and adhesion	[230]
NAD(P)H-dependent quinone oxidoreductase 1 (NQO1)	Fuchs endothelial corneal dystrophy	HCEC-21 T cells	KO	Promotion of EMT via an increase in intracellular ROS levels, and expression of Snail1, fibronectin, and N-cadherin	[231]

Table 1 (continued)

Target genes	Disease	Models	Methods	Major outcomes	References
Anterior gradient 2 (AGR2)	Lung cancer	A549 cells	KO	Induction of EMT phenotype similar to that induced by TGF- β treatment	[232]
Cell adhesion molecule 1 (CADM1)	Lung cancer	A549 cells	KO	Reduction in EMT-induced susceptibility to natural killer (NK) cytotoxicity	[233]
Double-stranded RNA-specific endoribonuclease 1 (DICER1), Fibronectin 1 (FN1), Forkhead box P1 (FOXP1), TGFB1	Lung cancer	A549 and MCF-10A cells	KO	Cancer cells lowers the energy barrier of the EMT to MET transition by modulating ceRNA levels	[234]
Fibroblast growth factor receptor 1 (FGFR1) and ZEB1	Lung cancer	HCC827 and PC9	Induction	Increase in ZEB1, but not FGFR1 expression, is a driver of EMT	[235]
Phosphatase and tensin homolog (PTEN)	Lung cancer	A549 and NCI-H460 cells	KO	Induction of EMT by nuclear translocation of β -catenin and Snail/Slug, increase proliferation, migration and invasiveness	[236]
Fyn-related kinase (FRK)	Lung cancer	H1299 cells	KO	Inhibition of EMT and metastasis	[151]
FGFR1	Lung cancer	HCC827, HCC4006, H1975, MGH707-1, MGH174-2A, MGH721-1, MGH792-1A, MGH700-2D and mice bearing PC9 xenograft tumors	KO	Inhibition of survival and EMT-associated acquired drug resistance	[237]
Prostate-derived Ets factor (PDEF)	Gastric cancer	AGS cells	KO	Inhibition of cell proliferation, migration and invasion	[238]
Polymerase I and transcript release factor (PTRF)	Melanoma	MM000921 and MM121224	KO	Reduction in invasive index and decreased ability to form spheres in 3D culture	[239]
RUNX family transcription factor 2 (RUNX2) [Runt domain]	Melanoma	A375 cells	KO	Reduction in cell proliferation, increased apoptosis, and reduced EMT phenotype	[240]
Runx2 [Runt domain]	Melanoma	A375 cells	KO	Inhibition of Runx2 DNA-binding function and reduced angiogenesis	[241]
Interleukin-6 (IL-6)	Head and neck squamous cell carcinomas (HNSCC)	Primary human dermal microvascular endothelial cells (HDMVEC)	KO	Impaired tumor growth and reduced cancer stem cell fraction	[242]
Lysine demethylase 6B (KDM6B)	Glioma	U87 and U251	Interference and activation	KDM6B promotes proliferation, migration and invasion by increasing the expression of SNAIL	[243]

Table 1 (continued)

Target genes	Disease	Models	Methods	Major outcomes	References
TEA domain transcription factor 1 Or 4 (TEAD1/4)	Glioblastoma	Patient-derived GBM lines	KO	Reduced migration and altered migratory and EMT transcriptional signatures	[244]
Vascular endothelial growth factor (VEGF) A	Barrett esophagus	Barrett esophagus cell lines (BAR-T)	KO	Inhibition of EMT by downregulation of ZEB1/2	[245]
Mixed-lineage leukemia 2 (MLL2)	Esophageal squamous cell carcinoma	Eca109 cells	KO	Inhibition of cell proliferation and migration ability, induced cell cycle arrest at G1 stage and restraint of EMT	[246]
ZEB1	Posterior polymorphous corneal dystrophy (PPCD)	Corneal endothelial cells (CEEnCs)	KO	Inhibition of proliferation and migration capacity	[247]
Epithelial membrane protein 2 (EMP2)	Cardiogenic differentiation	E14Tg2a mouse embryonic stem cells (mESCs)	KO	Upregulation of pluripotency markers, defective gastrulation and deregulated EMT process	[248]
Mesoderm posterior bHLH transcription factor 1 (MESP1)	Early cardiovascular tissue differentiation	Human induced pluripotent stem cells (hiPSC)	KD	Inhibition or delay of vascular specification and defective EMT during vascular progenitor differentiation	[249]
BCL2 related protein A1 (BFL-1) and BCL2 like 2 (BCL-w)	Various human cancers	TYKNU, WiDR and A549	KO	Identified BCL-2 family members as potent drivers of intrinsic resistance and drives EMT-induced resistance to apoptosis	[250]
PTEN	Mouse mammary gland morphogenesis	Blastocysts (in vivo)	Single nucleotide mutation	Inhibition of EMT markers TWIST1, SNAIL and YAP1	[251]

Conclusion

EMT is a complex process that is involved in diverse cellular functions both physiologically and pathologically. Numerous genes have roles in the initiation or progression of this critical process. The CRISPR/Cas technology has already contributed to enhance our understanding of this complex process. The use of this technology to knockout, repress or activate genes will help to identify more EMT-related genes and the downstream cellular pathways and functions they regulate. In addition, the ability to effectively modulate EMT and MET will further contribute to understanding their physiological roles and pathological consequences. The use of CRISPR/Cas technology shall facilitate high-throughput screening of gene function in EMT-related cellular processes.

EMT-related pathways have been the targets of some clinical trials [252]. CRISPR technology is also an emerging area in clinical trials, with few studies recently initiated or currently recruiting patients. Most of these studies will test targeting programmed cell death protein-1 (PD-1) to overcome cancers, such as Phase I clinical trial of infusing back into patients the PD-1 knockout T cells in the laboratory by CRISPR/Cas9 for treatment of metastatic non-small cell lung cancer (NCT02793856). CRISPR/Cas9 technology has not yet been applied to target EMT-related genes in clinical studies, however with the increasing use of CRISPR/Cas9 in clinical trials, EMT-related genes may be a future target of this technology in clinical investigations. Meanwhile, further preclinical studies need to be implemented to investigate the potential on- and/or off-target toxicity of this approach as well as its efficient targeted delivery.

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