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The epigenetics of epithelial-mesenchymal plasticity in cancer

Wai Leong Tam[1,2] and **Robert A Weinberg**[1,2,3]

[1]Whitehead Institute for Biomedical Research, Cambridge, Massachusetts, USA

[2]Massachusetts Institute of Technology (MIT) Ludwig Center for Molecular Oncology, Cambridge, Massachusetts, USA

[3]Department of Biology, MIT, Cambridge, Massachusetts, USA

Abstract

During the course of malignant cancer progression, neoplastic cells undergo dynamic and reversible transitions between multiple phenotypic states, the extremes of which are defined by the expression of epithelial and mesenchymal phenotypes. This plasticity is enabled by underlying shifts in epigenetic regulation. A small cohort of pleiotropically acting transcription factors is widely recognized to effect these shifts by controlling the expression of a constituency of key target genes. These master regulators depend on complex epigenetic regulatory mechanisms, notably the induction of changes in the modifications of chromatin-associated histones, in order to achieve the widespread changes in gene expression observed during epithelial-mesenchymal transitions (EMTs). These associations indicate that an understanding of the functional interactions between such EMT-inducing transcription factors and the modulators of chromatin configuration will provide crucial insights into the fundamental mechanisms underlying cancer progression and may, in the longer term, generate new diagnostic and therapeutic modalities for treating high-grade malignancies.

The neoplastic cells within individual human carcinomas reside within a spectrum of phenotypic states, ranging from a fully differentiated epithelial state to a dedifferentiated mesenchymal state, each of which is associated with distinct functional traits. While they reside within primary tumors, the bulk of carcinoma cells generally exhibit predominantly epithelial characteristics. However, in order to invade, disseminate to distant tissues and subsequently form metastatic colonies, neoplastic epithelial cells must shift, at least transiently, into a more mesenchymal phenotype. This shift is achieved by the activation of the complex cell-biological program termed the EMT. During an EMT, carcinoma cells shed their differentiated epithelial characteristics, including cell-cell adhesion, polarity and lack of motility, and acquire mesenchymal traits, including motility, invasiveness and, importantly, many of the attributes of stem cells[1,2].

In normal and neoplastic epithelial tissues, it seems that the physiologic activation of EMT programs depends on the convergence of multiple signals that a cell receives from its nearby

Correspondence should be addressed to: R.A. W. (weinberg@wi.mit.edu).

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microenvironment. Various paracrine signaling factors can trigger the induction of an EMT program, doing so by activating a corresponding diverse array of intracellular signaling cascades[3–5] (Box 1 and Fig. 1). In response, a cohort of EMT-inducing transcription factors (EMT-TFs) becomes expressed and functionally activated[6,7]. The forced expression of individual EMT-TFs, such as TWIST, SNAIL, SLUG or ZEB1, has been found to activate EMT programs in epithelial cells, and their elevated expression has been well documented in invasive tumors[8–15]. Although the activation of an EMT program by individual extracellular signaling factors is possible in principle, it seems more likely that these afferent signals work in various combinations to provoke the expression of EMT-TFs and in turn the activation of EMT programs.

In carcinoma cells, the newly acquired mesenchymal traits resulting from activation of an EMT program endow these cells with the multiple features that are required to execute most steps of the invasion-metastasis cascade[1]. This cascade includes the ability of carcinoma cells to invade locally in the vicinity of primary tumors, intravasate, travel through the circulation, extravasate, survive in the parenchyma of a distant organ and form micrometastatic deposits, some of which may eventually form macroscopic metastases. This last step, termed ‘colonization’, probably involves the adaptation of carcinoma cells to foreign tissue microenvironments.

Importantly, this acquisition of mesenchymal attributes by carcinoma cells need not be permanent, as cells that have passed through an EMT while in the primary tumor may later revert to an epithelial state through a mesenchymal-epithelial transition (MET)[16,17], highlighting the plastic nature of these changes (Fig. 2). Indeed, within sites of dissemination, newly arrived carcinoma cells are unlikely to encounter the contextual signals that induced their precursors in the primary tumor to activate EMT programs; this may permit them to lapse back into an epithelial state. The reversibility of the EMT process involves widespread reprogramming of gene expression and implies that epigenetic regulators have important roles in this process, as discussed below.

The term ‘epigenetics’ has acquired multiple meanings in recent years[18]. Traditionally it has been used to describe the mechanisms that impose cellular phenotypes without concomitant changes in the genome of a cell, meaning without changes in its nucleotide sequences. More recently, however, the term has taken on a new meaning, as it has become apparent that epigenetic regulation is achieved in large part by the covalent modification of DNA, specifically the methylation of certain cytosine residues (that is, DNA methylation), as well as by the covalent modifications of the histone proteins that form DNA-associated nucleosomes. We refer here to the enzymes that catalyze these various biochemical reactions as epigenetic regulators.

Over the last decade, the generation of transcriptionally active and repressive histone marks that are catalyzed by a variety of histone-modifying enzymes has been recognized as a cornerstone of gene regulation[19]. For example, histone methyltransferases and demethylases can either add or remove methylation marks on the lysine residues of nucleosome subunits, especially those of histones H3 and H4. Acting combinatorially, these modifications help determine how DNA is packaged in chromatin, thereby dictating the transcriptional potential

of the underlying genes. More recent studies have revealed interesting links between EMT-TFs, which bind DNA in a sequence-specific manner, and the control of the chromatin configuration resulting from these histone modifications.

Phenotypic plasticity of cancer cells

The contributions of the EMT program in promoting cancer cell invasion and metastasis have been well documented in many types of carcinoma, including those arising in the breast, prostate, colon, head and neck, ovary and lung[2,20]. In addition, such a program has been found to generate cells that either exhibit stem-like properties or are poised to enter into the stem cell state[8,21–25]. This acquisition of stem-like characteristics holds important implications for the successful completion of the invasion-metastasis cascade by disseminated cancer cells. Passage through an EMT often imparts tumor-initiating properties to carcinoma cells, and these traits would seem to be crucial for the ability of disseminated cancer cells to serve as founders of new neoplastic colonies in anatomically distant sites. Of additional interest, diverse lines of evidence have increasingly indicated that the stem cell programs operating in carcinomas are quite similar to those that function in the corresponding normal cells of origin, that is, those residing in the tissues in which carcinomas initially arise[8,9,26].

The experimental observations described above are correlated with and supported by clinical evidence. High-grade tumors, notably those associated with poor patient prognosis, often contain cells that express molecular signatures that are associated with the expression of an EMT program. In breast cancer, for example, the expression of characteristically mesenchymal genes by carcinoma cells is typically enriched in the basal and triple-negative subtypes of tumors, both of which correlate with poor clinical outcomes[27]. Such tumors contain cells that behave as if they have undergone at least a partial EMT, with acquired expression of mesenchymal markers and retention of certain epithelial characteristics[28–30]. Cells isolated from these tumors also show many features of tumor-initiating cancer stem cells (CSCs), such as an enrichment for cells residing in the $CD44^{hi}CD24^{lo}$ antigenic state and a heightened resistance to diverse cancer therapies, as well as enhanced invasive and metastatic properties[2,20].

A spectrum of transitory cell states

The phenotypic plasticity of carcinoma cells to profoundly alter their behavior is not a contrivance of neoplasia but instead reflects transdifferentiation programs that play important parts in normal metazoan development and tissue repair[2,31]. For instance, the EMT program is activated very early during gastrulation and neural crest formation. Embryonic epithelial sheets that subsequently arise also undergo marked remodeling in either a reversible or irreversible manner, leading to the formation of the heart, musculoskeletal system, craniofacial features and peripheral nervous system[2]. During tissue repair in adult mammals, epithelial cells such as keratinocytes initially undergo an EMT and, after reconstitution of epithelial cell sheets, an MET[32], indicating that the reversible transitions between cell states are natural processes that are crucial to normal development and tissue homeostasis.

Although the EMT is often portrayed as simply a gain of clear-cut mesenchymal markers coupled with the complete loss of epithelial features, in reality it usually produces cells residing within a spectrum of intermediate phenotypic states. Stated differently, cells can advance to differing extents through an EMT program, progressively acquiring mesenchymal features as they shed epithelial ones; indeed, cells that have entered an EMT program rarely shed all of their pre-existing epithelial features. Accordingly, in the context of carcinoma pathogenesis, neoplastic cells may reside in a state in which they coexpress newly acquired mesenchymal markers together with retained epithelial ones—often termed a 'partial EMT'[33–37]. Normal and neoplastic cells may dwell only metastably in these intermediate states, being ostensibly primed to transition rapidly into cells expressing either fully epithelial or mesenchymal phenotypes. In carcinomas, these shifts are often and perhaps invariably instigated by contextual signals originating in the tumor microenvironment (the tumor-associated stroma).

Additional evidence of such phenotypic plasticity can be found in the cellular differentiation and dedifferentiation programs of normal epithelial stem cell compartments. Although stem cells within these niches differentiate to produce specialized progeny during development, a rare pool of stem cells must be continuously maintained through the process of self renewal. The traditional portrayal of the stem cell hierarchy indicates a unidirectional change from multipotent stem cells into more differentiated progeny. However, recent work has pointed to a deviation from this scheme: in particular, non-stem cells in the mammary gland seem to repopulate stem cells *in vitro* by dedifferentiation through mechanisms that remain unclear[38,39]. This has subsequently been shown to occur in the context of cancer during intestinal tumorigenesis *in vivo*[40]. Importantly, the observations that EMTs push differentiated epithelial cells toward a stem cell state and that EMT programs are activated in a variety of physiologic processes would seem on their own to indicate that such dedifferentiation is indeed part of the behavioral repertoire of normal epithelial cells and, by extension, their neoplastic derivatives.

It has been proposed that the epithelial differentiation program is a default pathway for cells in a mesenchymal state[41]. In the absence of signals that continuously reinforce residence in the mesenchymal or stem cell state, the mesenchymal products of EMT may naturally revert to an epithelial state (Fig. 3). Implicit in this hypothesis is the notion that residence in the mesenchymal state must be actively and continuously supported by contextual signals. For example, a variety of epithelial cells respond, at least transiently, to transforming growth factor-β (TGF-β) signaling by activating the expression of mesenchymal genes. However, after withdrawal of TGF-β, such cells revert back to an epithelial state. In contrast to this model of ongoing dependence, there is also evidence that CSCs can maintain their own residence in the mesenchymal state metastably through the activation of autocrine signaling loops that seem to liberate them from dependence on continuous paracrine EMT-inducing signals originating elsewhere within tissues[3,42].

Interactions between epigenetic and transcription regulators

E-cadherin (encoded by the *CDH1* gene) forms a keystone of the epithelial state, and the downregulation of this adherens junction protein represents a hallmark of passage through

an EMT program. This explains why its expression must be precisely regulated. A number of studies have demonstrated that several EMT-TFs become recruited to the promoter of *CDH1* after activation of an EMT program, where they repress its transcription[11]. However, the precise role of epigenetic regulators in facilitating and stabilizing these changes is less well understood. More recent studies have revealed that the epigenetic silencing of E-cadherin is highly complex and orchestrated by a variety of histone-modifying enzymes that cooperate to confer various degrees of repression of the *CDH1* promoter.

Polycomb repressor–mediated silencing

The polycomb group (PcG) proteins constitute a group of epigenetic regulators that have a key role in regulating the expression of E-cadherin. They function as transcription repressors by directing lineage choices during early development and stem cell differentiation[43,44]. For example, they help to preserve the repression of homeotic genes during development, thereby ensuring that progenitor or stem cells remain in an undifferentiated state[45]. Importantly, PcG proteins are also capable of driving tumor development by controlling the phenotypic states of cancer cells.

The PcG proteins assemble with other scaffolding proteins to form the multisubunit polycomb repressive complexes (PRCs), which silence transcription by modifying histones and recruiting a variety of additional repressors[46]. Two distinct classes of polycomb complexes, PRC1 and PRC2, participate in promoting the EMT but do so in different ways. Typically, PRC2 is initially recruited to target genes, which may then be followed by PRC1 recruitment[43,47]. Enhancer of zeste homolog 2 (EZH2), which functions together with suppressor of zeste 12 homolog (SUZ12), is a PRC2 subunit that catalyzes the trimethylation of K27 on histone H3 (H3K27me3) in the nucleosomes surrounding promoters, thereby leading to transcriptional repression[48].

During cancer pathogenesis, the elevated expression of certain PRC2 subunits is thought to drive malignant progression through an EMT program[49–51]. This is attributed, in part, to their ability to repress key genetic targets, including *CDH1*, that are otherwise essential for enforcing the epithelial state of neoplastic cells. The manner in which PRC2 can be recruited to certain target genes is not well understood but seems to involve physical interactions between PRC2 and certain sequence-specific transcription factors. Many of the EMT-TFs contain DNA-binding domains that recognize the enhancer box (E-box) nucleotide sequence motifs present on certain gene promoters[11]; this recognition confers the specificity required for transcription factors to become localized to specific genomic targets. For example, in pancreatic and colon adenocarcinoma cells, SNAIL is associated with the *CDH1* promoter and physically interacts with EZH2 and SUZ12 to catalyze the trimethylation of H3K27 in nearby nucleosomes, thereby silencing *CDH1* gene transcription[50]. Subsequently, the ongoing repression of *CDH1* seems to be dependent on the continuous presence of SNAIL[50]. Hence, sequence-specific master transcription factors (in this case EMT-TFs) are initially recruited to key target loci; their continued presence enables the subsequent recruitment of chromatin-modifying enzymes, providing one model for coupling transcription control with the epigenetic regulatory mechanisms that govern the EMT[52] (Fig. 4).

Several lines of evidence have begun to shed light on the clinical importance of PRC2 in the pathological EMTs that occur in certain carcinomas. For example, basal breast cancers and *BRCA1*-deficient tumors, both of which bear EMT gene expression signatures[28–30], tend to overexpress *EZH2* (refs. 53–57). It is possible that EZH2 enforces the silencing of E-cadherin expression in subpopulations of carcinoma cells within these mammary tumors. Indeed, loss of E-cadherin on its own can drive certain epithelial cells toward a mesenchymal state[58]. Similarly, elevated *EZH2* expression has been detected in aggressive forms of bladder and prostate tumors and is correlated with loss of *CDH1* expression[49]. Still, these observations are at best correlative and do not demonstrate directly that EZH2 promotes the mesenchymal cell states of these carcinoma cells by driving the trimethylation of the H3K27 residues that are associated with the *CDH1* promoter.

The initial formation of H3K27me3 by PRC2 facilitates the subsequent recruitment of chromodomain-containing proteins, which recognize and bind to previously methylated histones; examples of these proteins are the CBX2, CBX4 and CBX8 subunits of PRC1 (ref. 59). PRC1 contains another functionally important subunit, the polycomb ring finger oncoprotein, BMI1, whose expression is dysregulated in many cancers[60–63]. In recent years, BMI1 has also been characterized as a stem cell factor that drives CSC function, and its upregulation strongly correlates with invasive tumor phenotypes[64–67]. Given the increasing recognition that CSCs within carcinomas exhibit components of the EMT program, it is plausible that BMI1 plays a key part in facilitating the cell-state transitions that lead to the formation of more mesenchymal, CSC-like cells.

In nasopharyngeal carcinomas, *BMI1* overexpression on its own induces a mesenchymal-like phenotype and thus enhances the invasiveness and motility of the associated neoplastic cells[68]. The precise mechanism by which BMI1 mediates the repression of target genes is not well understood. It is clear, however, that BMI1 can transcriptionally downregulate expression of the tumor suppressor *PTEN*, which in turn leads to activation of phosphoinositide 3-kinase (PI3K)-AKT signaling and post-translational stabilization of SNAIL[68]; the resulting accumulation of SNAIL presumably facilitates the activation of EMT programs. Another line of evidence suggests that the elevated expression of BMI1 may in fact be directly linked to the EMT program, as TWIST has been found to bind the *BMI1* promoter and upregulate its expression. Thus, both TWIST and BMI1 seem to be essential for the execution of EMT programs and the acquisition of the CSC phenotype[69,70].

Histone deacetylases and silencing

Acetylation of lysine residues is another avenue leading to histone modification. Histone acetylation is often associated with transcription activation, whereas deacetylation results in repression. Histone acetyltransferases add the acetyl group to several distinct lysine residues, such as the K9 and K14 residues of histone H3, whereas histone deacetylases (HDACs) catalyze their removal. Similar to the PcG proteins, histone acetyltransferases and HDACs form multimeric complexes. During metastasis, HDAC1 and HDAC2, which function as components of the Mi-2–nucleosome remodeling and deacetylase (NuRD) repressive complex, are recruited by mouse Snail to the *Cdh1* promoter and contribute to its

silencing[71,72]. Treatment with an HDAC inhibitor, Trichostatin A, blocks the repressive effects of Snail and prevents metastasis[71].

This ability of Snail to silence target genes seems to be conferred by its N-terminal SNAG domain, which helps recruit transcription repressors such as HDACs and Sin3A. Accordingly, Snail mutants containing a deletion or truncation of the SNAG domain are unable to repress E-cadherin expression[71,73]. Additionally, TWIST can associate directly with the NuRD complex to silence E-cadherin in human and mouse breast cancer cells but seems to recruit NuRD in a manner that is distinct from that of Snail, doing so through its ability to recognize and bind other subunits of the NuRD complex, such as Mi-2b and MTA2 (ref. 74).

Histone demethylases: an emerging class of EMT regulators

More recent studies have begun to link the lysine-specific demethylase LSD1 to EMT and cancer progression. LSD1 was the first histone demethylase to be identified and was initially shown to remove methyl groups from the transcription-activating H3K4me3 mark. LSD1 does this through its amine oxidase domain, which catalyzes oxidation of biogenic amines, including the N terminus of methylated histones, resulting in gene repression[75]. In mixed-lineage leukemia cells, LSD1 regulates leukemia stem cell potential by blocking differentiation and apoptosis[76]. Among breast cancers, LSD1 is highly expressed in estrogen receptor-negative tumors, which tend to bear mesenchymal gene signatures[77], pointing to its possible involvement in promoting the EMT. Indeed, SNAIL-driven EMT of human mammary epithelial cells involves the recruitment of LSD1, which it employs to silence epithelial genes, including those encoding E-cadherin, claudins and cytokeratins[78]. This interaction between LSD1 and SNAIL depends on the amine oxidase domain of LSD1 and the SNAG domain of SNAIL. Interestingly, the SNAG domain seems to share sequence similarities with the N terminus of histone H3, which enables LSD1 to recognize the SNAG domain, leading to the formation of LSD1-SNAIL complexes on gene promoters[73].

These experimental results, together with the clinical observations that LSD1 overexpression is correlated with poor survival in several other types of cancer, have prompted investigations into the therapeutic utility of LSD1 inhibitors. In mixed-lineage leukemic cells, pharmacologic targeting of LSD1 decreased their leukemia-initiating ability and increased the re-expression of genes that are associated with myeloid differentiation[76,79]. These effects were also accompanied by a concomitant increase of H3K4me2 at those promoters[79]. Treatment of breast cancer cells strongly expressing LSD1 with pharmacologic inhibitors targeting amine oxidases conferred growth inhibition and led to a global increase of H3K4me3 (ref. 77).

Of note, other findings seem to directly contradict these reports of the oncogenic properties of LSD1. Some studies have found that LSD1 can inhibit the invasiveness of breast cancer cells and suppress their metastatic potential[80]. These contradictory observations of the functions of LSD1 may be attributed to the multiple histone lysine substrates it is able to modify. Apart from converting active H3K4me2 or H3K4me3 to the less active H3K4me1 mark, LSD1 is now known to cause demethylation of the inactive H3K9me3 mark, converting it into the less repressive H3K9me1 or H3K9me2 marks, thereby causing gene

derepression[81]. Taken together, the functional outcome of LSD1 activity depends on the balance between the activation and repression of different subsets of genes, and these conflicting functions seem to derive from its ability to modify either H3K4me3 or H3K9me3 or both. Hence, the design of therapeutic strategies aimed at targeting LSD1 will need to respond to its known pleiotropic actions.

Bivalent histone modification and poised transcription

The permissiveness of chromatin loci to gene transcription is initially regulated through the acquisition of a variety of epigenetic modifications and is subsequently consolidated through higher-order changes in the chromatin architecture. These higher-order changes involve the formation of euchromatin (containing actively transcribed genes) or heterochromatin (containing repressed genes) domains. Importantly, certain segments of the DNA may be associated with facultative heterochromatin, implying an ability to alternate between induced and repressed states of expression; this behavior contrasts with the known behavior of constitutive heterochromatin, which is associated with permanently silenced genes. H3K27me3 has been found to be associated with facultative heterochromatin that can be converted readily into an active euchromatic state[46]. In embryonic stem cells, for example, the coexistence of both the H3K27me3 and H3K4me3 modifications on certain promoters marks the existence of genes in a ‘bivalent’ state that are not being actively transcribed. Genes residing in this configuration are nevertheless poised to become activated in response to specific differentiation cues that lead to the removal of the repressive H3K27 methylation mark[82]. This finding indicates that bivalent genes are not stably repressed but instead remain responsive to dynamic regulation by certain physiologic signals.

Indeed, cancer cells that are phenotypically plastic seem to contain bivalent modifications at the *ZEB1* promoter, which facilitate their rapid dedifferentiation to a stem-like state[83]. Bivalent promoters are also observed in certain cancer cells that exhibit stem cell-like properties. Within the $CD44^+$ stem cell-enriched populations of primary human mammary epithelial tissues, the *CDH1* promoter bears the bivalent H3K4me3 and H3K27me3 modifications while being silenced, whereas the more differentiated $CD24^+$ cells that express abundant E-cadherin carry only the active H3K4me3 mark[84]. This chromatin configuration in $CD44^+$ stem cells should logically permit their rapid differentiation into a $CD24^+$ epithelial state through loss of the repressive H3K27me3 mark on the *CDH1* gene and, quite possibly, other epithelial-specific genes. Likewise, the *TWIST* and *FGF2* genes, although silent in $CD24^+$ non-stem cells, seem to carry some bivalent features, whereas $CD44^+$ cells expressing these same genes contain only the H3K4me3 activating modification[84]. These observations may indicate that at least some of the $CD24^+$ non-stem cells are poised to become dedifferentiated into $CD44^+$ stem cells[38].

The bivalent configuration of certain EMT-associated genes presumably permits the dynamic regulation of gene expression and contributes to the plastic nature of these mammary epithelial cells. Nevertheless, it remains unclear whether the modulation of cellular plasticity depends largely on changes in bivalent genes and whether such bivalent genes are commonly found throughout the genomes of cells known to exhibit plasticity. Of note, time-lapse microscopy has revealed that epithelial cells can interconvert rapidly and

reversibly between epithelial and mesenchymal cell phenotypes[14,85]. In these cases, cell division seems to be dispensable for the observed cell-state transitions, underscoring the need for rapid, dynamic changes in epigenetic regulation at the level of chromatin, ostensibly involving substantial shifts in histone modification.

Stable repressive marks and long-term silencing

As indicated above, bivalent genes are not associated with long-term, durable silencing, as they need to respond rapidly and reversibly to contextual signals. In contrast, during certain steps of the invasion-metastasis cascade, epithelial genes must be repressed for extended periods of time. For example, in order for invading cells to detach from and remain dissociated from primary tumor masses, their metastable residence in a quasi-mesenchymal state (involving the stable silencing of certain epithelial genes) needs to be enforced during the extended invasion period.

Such stable repression may be explained by the special properties of certain histone modifications. Thus, the trimethylation of H3K9 creates constitutive heterochromatin that is more resistant to the activation of transcription than is the chromatin resulting from the H3K27me3 modification. SNAIL was recently found to associate with G9a (also known as EHMT2), a major histone methyltransferase that is responsible for creating the H3K9me2 repressive mark[86]. Subsequently, the addition of a third methyl group to H3K9 by SUV39H1 (another histone methyltransferase) leads to a H3K9me3 modification that confers a more stable and durable repressive state than H3K9me2. In fact, SNAIL also interacts with SUV39H1 during EMT induced by TGF-β or SNAIL and mediates silencing of the *CDH1* promoter[87]. Consistent with its role in silencing epithelial genes, SUV39H1 seems to be more abundantly expressed in the mesenchymal, basal subtype of breast cancer cells relative to the more epithelial cells of the luminal subtype[87].

The formation of the H3K9me3 mark in promoter-associated chromatin has important consequences for long-term gene silencing, as this modification is thought to be a prelude to the recruitment of DNA methyltransferases (DNMTs) that catalyze DNA methylation[46]. Methylation at the CpG dinucleotides near gene promoters is associated with highly stable gene silencing that can be inherited with high fidelity over the course of multiple successive cell divisions. As a result, the progenies of a cell that initially underwent a specific CpG methylation continue to exhibit mesenchymal features that may in turn support their ability to invade or disseminate. Indeed, in the claudin-low subtype of human breast cancers, which is one of the most mesenchymal subtypes of this disease, SNAIL has the ability to recruit G9a and DNMT to the *CDH1* promoter, resulting in DNA methylation and thus the stable shutdown of E-cadherin expression[86].

To summarize, the involvement of various histone-modifying enzymes and the modifications they create is consistent with the view that the EMT program subsumes a succession of changes as cells pass from a fully epithelial to a fully mesenchymal state rather than a single coordinated change in cell phenotype. At the level of chromatin, these cell-biological changes may be accompanied by, and indeed driven through, a spectrum of progressively more stable epigenetic changes that control passage through distinct phases of an EMT (Fig. 3). For instance, the repression of epithelial genes may involve an initial gain

of H3K27me3 to form a bivalent modification with H3K4me3 (ref. 88), creating a highly plastic state that is reversible. This may be succeeded by the loss of H3K4me3, which facilitates the subsequent formation of constitutive heterochromatic H3K9me3 modifications that are acquired more stably. Subsequently, in the sustained presence of potent EMT-inducing signals, H3K9me3 sets the stage for the recruitment of DNMTs, which proceed to methylate the DNA of gene promoters that confer epithelial traits, creating the highly stable CpG dinucleotides that can be perpetuated over many cell generations.

Genome-wide epigenetic reconfigurations

Most chromatin modification studies, such as those discussed above, have focused on a small number of gene promoters that are epigenetically regulated. These studies do not, however, address how the epigenetic ‘landscape’ of the entire genome (that is, the epigenome) is altered during transitions between cell states. Active genome-wide epigenetic reconfiguration is crucial for normal development, differentiation and disease, and chromatin-modifying enzymes are indeed rarely targeted to small sets of gene promoters. Such widespread modification of histones often results in marked changes to the overall chromatin structure and hence expression changes of large constituencies of genes within a cell. Moreover, recent studies have found that ‘long-range’ epigenetic remodeling, through either the activation or repression of large genomic domains, can be a major driving force in tumorigenesis[89,90].

Typically, long-range epigenetic silencing has been thought to involve the gain of repressive histone modifications, such as deacetylation and K9 and K27 trimethylation, as well as DNA methylation across domains spanning up to several megabases; these regions may contain tumor-suppressor genes or those associated with the regulation of an epithelial cell state[90,91]. More recently, an alternative mechanism of such domain gene deregulation that also operates through long-range epigenetic activation has been described in certain cancer cell types. Such regions, which are characterized by a gain of active chromatin marks and a loss of repressive marks, tend to contain oncogenes, microRNAs and cancer biomarkers[89,92].

In the context of cellular plasticity, it remains unclear whether EMT-associated genes are also epigenetically regulated as parts of large genomic domains. We imagine that long-range epigenetic remodeling may not directly drive epithelial or mesenchymal cell-state transitions on its own. However, it may create a permissive cell state that allows for the subsequent, more focused actions of EMT effectors, doing so by altering the overall epigenome of epithelial cells. Consequently, we note that currently embraced, simpler schemes of local chromatin regulation that involve the directed actions of EMT-TFs on a small cohort of key target genes will need to be revised in the future as the complexities that are associated with the large-scale remodeling of the epigenetic landscape become apparent.

Epigenetic therapies targeting EMT

During cancer development, a rare population of CSCs is thought to drive tumor initiation, recurrence and metastasis[93-96]. Such cells, which tend to exhibit certain mesenchymal traits, are also more resistant than bulk non-CSCs to attack by a variety of therapeutic agents and

therefore create a substantial obstacle to achieving favorable clinical responses. The discovery of the molecular links between epigenetic regulation, the CSC state and epithelial-mesenchymal plasticity may reveal new targets for therapeutic intervention, more specifically by suggesting treatments that can target the more mesenchymal CSCs.

For example, restoring the expression of epithelial-associated regulators, including certain microRNAs (Box 2 and Fig. 5) that help promote the differentiation of CSCs into an epithelial state, might be achieved by epigenetic therapies. The DNA demethylating agent 5-azacytidine (5-azaC) seems capable of restoring the expression of the epithelial-specific microRNA miR-200 (refs. 97,98) and may therefore be useful for sensitizing CSCs to traditional therapeutic agents by inducing their differentiation. Likewise, inhibitors that target the activity of the histone deacetylase sirtuin 1 (SIRT1) may help promote E-cadherin or miR-200 expression[99,100]. Treatment of leukemic cells with 5-azaC, trichostatin A or both seems to halt leukemia progression, an effect that is attributable in part to the induction of cellular differentiation or the reactivation of tumor-suppressor genes[101–103]. Both of these single agents have now been applied in some clinical settings as part of a combined therapy for treating myelodysplastic syndrome and leukemia[101,103,104].

Despite the apparent clinical efficacy of such epigenetic therapies in a limited number of cancers, the long-term effects of these drugs on normal cell physiology and tissue function remains a matter of debate. Although these agents may act through affecting certain targeted genes, their impact on the expression of countless other untargeted genes cannot be readily assessed, which complicates attempts to reduce side-effect toxicities. For example, some studies have noted that DNA hypomethylation in mice deficient for Dnmt1 activity promotes chromosome instability and increases tumor incidence[105–108]. Furthermore, such epigenetic modulators could have conflicting pathological consequences depending on when they are applied after the initiation of cancer formation: during the early stages of tumor progression, these agents may cause mesenchymal cells to differentiate within the primary tumor or arrest their ability to undergo cell-state transitions; later on they may promote metastatic colonization by acting on carcinoma cells that have already disseminated to distant organs.

Although HDAC inhibitors have been useful in treating certain hematological tumors, thus far they have exhibited limited impact on solid tumors, notably carcinomas. Such inhibitors also seem to have conflicting effects on regulating cell-state transitions. Earlier reports have suggested a role for HDACs, especially HDAC1 and HDAC2, in promoting EMT in a TGF-β–dependent manner. Indeed, the inhibition of HDAC activity has been found to block cell-state transitions in hepatocytes as well as head-and-neck squamous carcinoma cells[109–111]. However, more recent studies have found that HDAC inhibitors can induce an EMT in prostate and nasopharyngeal cancer cells[112,113]. Hence, the utility of these treatments for carcinomas remains unclear and will need to be evaluated carefully.

Thus far, the majority of epigenetic drugs have been aimed at inhibiting the epigenetic ‘writers’ and ‘erasers’, which are the enzymes that attach or remove, respectively, the covalent marks on various histones. More recent strategies have been applied to targeting epigenetic ‘readers’, which recognize and then bind certain modified histones. Interestingly,

insights into the ability of one such inhibitor to target epigenetic landmarks in a cell state– or type–specific manner arose from the marriage of two apparently unrelated findings. First was the success in the development of the JQ1 cell-permeable small molecule, which binds to acetyl-lysine recognition motifs (termed bromodomains) and thereby inhibits the function of the bromodomain-containing transcriptional coactivator BRD4 (refs. 114,115). Later came the recognition that the simultaneous binding of multiple master transcription factors and the Mediator coactivator complex to certain genomic loci creates so-called 'super enhancers' at key cell-identity genes; indeed, BRD4 is among the proteins that has been found to bind to such super enhancers[116,117]. Super enhancers are localized to unique, relatively small subsets of genes that differ between cell states, and the loss of a single crucial component, such Mediator or BRD4, from these complexes can cause super enhancer–mediated gene expression to be lost. For example, super enhancers are often found at key oncogenes, such as *MYC*, and the inhibition of BRD4 may preferentially affect the function of MYC and potentially target tumor cells in a highly selective manner[117]. Along these lines, it is plausible that cells residing in either an epithelial or mesenchymal state carry distinct sets of active super enhancers in their genomes. The identity of the genes that are regulated by such super enhancers may provide clues into key regulatory landmarks that differ between cell states. Hence, the inhibition of bromodomain-containing proteins could potentially destabilize the continued residence of carcinoma cells in a mesenchymal state, which may rely on the activity of super enhancer–driven genes to maintain their long-term residence in this state.

Perspectives

The development of new epigenetic therapies to target carcinomas will require more comprehensive descriptions of the epigenetic profiles that distinguish mesenchymal CSCs and epithelial non-CSCs. A number of epigenetic enzymes and histone readers, such as bromodomain- and chromodomain-containing proteins, seem to be differentially expressed between cell states (W.L.T., unpublished data); this observation suggests a dependency of CSCs on certain proteins that might be targeted with small molecules that are designed to act against these proteins specifically[118,119]. Indeed, a similar strategy has been implemented for the specific targeting of CSCs, which depend more strongly on certain kinase signaling networks, and resulted in the selective elimination of mesenchymal CSCs while having little impact on the bulk epithelial fraction, which in principle can be eliminated by use of conventional therapeutic agents[120, Tam et al.,2013. http://dx.doi.org/10.1016/j.ccr.2013.08.005].

Over the next several years, chromatin-modifying enzymes will likely be implicated in the transcriptional regulation of epithelial and mesenchymal cell–state programs and thus in the regulation of the EMT. It is also likely that the actions of these various regulators will be closely tied to those of EMT-TFs. Together with the currently achievable, precise mapping of genome-wide localizations of transcription factors, chromatin modulators, histones and DNA modification marks, it is probable that high-resolution, genome-wide transcription and epigenetic blueprints that underlie the organization and expression of EMT programs will be produced in the near future. These maps will likely reveal previously unidentified landmarks within the genome, such as conserved regulatory sequence elements and those occupied by

new transcriptional regulators, including cofactors that are involved in various aspects of transcriptional regulation.

Importantly, and as emphasized here, it is becoming increasingly evident that the EMT program does not operate in a simple binary fashion, controlling the alternation of cells between two extreme cellular states. Instead it is now realized to be a highly dynamic process that involves a series of transitions and a spectrum of multiple intermediate states lying between these two endpoints. The interconversion of cells between these alternative states, resulting in the observed phenotypic plasticity, depends on the modulation of epigenetic regulatory mechanisms that remain poorly understood. Accordingly, the studies of the EMT that have previously focused on these extreme states must now begin to elucidate in a more nuanced fashion the regulators orchestrating these intermediate states, which seem to be more typical of the cells within actual human carcinomas. The resulting information will likely provide valuable insights into some of the early events that occur during the initiation of the EMT and MET programs and quite possibly reveal clues about the types of contextual signals that are involved. We foresee that these signals, notably those originating in the microenvironment near carcinoma cells, will be found to play key parts in governing the advance of carcinoma cells through EMT programs, thereby determining tumor progression and clinical outcomes of patients with cancer.

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Box 1

Autocrine and paracrine signaling networks for the maintenance of an EMT program

Various extracellular ligands activate and maintain the EMT program, operating in either an autocrine or paracrine manner (reviewed in refs. 5,131,132). TGF-β signaling is a major inducer of EMT during embryonic development and cancer progression[133,134]. TGF-β signals through two distinct receptor serine/threonine kinases, TGF-βR1 and TGF-βR2, which then phosphorylate the cytoplasmic SMAD2 and SMAD3 proteins. Activated phosphorylated SMAD2 and SMAD3 form complexes with SMAD4, which then translocate into the nucleus to regulate genes that are important in the control of cell fate. In various carcinomas, TGF-β signaling is commonly hyperactivated and promotes invasion and metastasis[135–137]. Hence, inhibition of TGF-β signaling has the potential to block the induction of EMT programs and, therefore, disease progression. TGF-β signaling has also been demonstrated to result directly in the epigenetic regulation of downstream target genes. For instance, SMAD2 and SMAD3 associate with certain epigenetic regulators, such as tripartite motif containing 33 (TRIM33), which displace repressive histone modifications, thereby creating a poised chromatin structure that can be accessed by transcriptional regulators[138]. In addition, exposure of mouse hepatocytes to TGF-β can reduce the bulk amounts of the heterochromatic H3K9me2 mark and increase the amounts of the H3K4me3 euchromatic and H3K36me3 transcription elongation marks[139]. The gain of these activating modifications seems to be crucial for EMT-mediated phenotypes such as cell motility.

WNT signaling is another developmentally important pathway that becomes dysregulated in a wide variety of carcinomas and contributes to the expansion and maintenance of CSCs in these tumors[140–143]. During mouse embryonic development, WNT activates β-catenin activity, which is necessary for gastrulation—an EMT-driven process[144,145]. Hyperactive WNT signals can trigger EMT-like programs, resulting in the aberrant activation of the β-catenin–TCF cascade and tumor progression[3,146,147]. EMT in breast cancer cells may be mediated through the stabilization of SNAIL activity as a result of WNT activation or by the transactivation of the mesenchymal marker vimentin by the b-catenin–TCF complex[148,149].

Various receptor tyrosine kinases that are activated by hepatic growth factor, epidermal growth factor, fibroblast growth factor and PDGF ligands can also contribute to the expression of EMT programs in a context-dependent manner that varies across different carcinomas[42,150–153]. Numerous studies have demonstrated that mesenchymal cancer cells depend on elevated expression of these signaling molecules for the induction and maintenance of an EMT transcriptional program.

Although the types of signaling network that guide the EMT are fairly well defined, the manner by which their downstream effector proteins, such as SMAD and b-catenin, feed into the induction of an EMT program is less well understood. Presumably they mediate the transcriptional activation of pleiotropically acting EMT transcription factors. Certain functional response elements, such as SMAD-binding elements, TCF-LEF binding sites,

E-boxes and activator protein 1 (AP-1) sites found within the promoters of the *SNAIL* and *SLUG* genes, may provide entry points for responding to extracellular signals (W.L.T., unpublished data). For instance, during neural crest development in the mouse embryo, bone morphogenetic protein–dependent activation of Smad1 results in its recruitment to the *Slug* promoter and leads to its precise temporal activation[154]. In invasive colorectal carcinoma where mutations of the adenomatous polyposis coli gene *(APC)* result in constitutive WNT signaling, the β-catenin–TCF4 complex is bound to the *ZEB1* promoter and upregulates its transcription[155].

Box 2

Microregulators of EMT

A repertoire of microRNAs maintains the epithelial phenotype by post-transcriptionally inhibiting mRNAs that encode EMT-TFs. The miR-200 microRNA family and miR-205 control the phenotypic state of epithelial cells through their interactions with *ZEB1* and *ZEB2* (refs. 128–130,156–158). During the early phases of tumor formation, most neoplastic cells within the primary tumor are epithelial; this state is enforced by the expression of the miR-200 family, which targets *ZEB1* and *ZEB2* mRNAs at numerous binding sites in their 3¢ untranslated regions[10]. After activation of an EMT program, the induction of ZEB1 and ZEB2 reciprocally represses the transcription of miR-200 microRNAs by directly repressing the *mir-200* promoter; the resulting loss of miR-200 relieves ZEB1 and ZEB2 inhibition, allowing maintenance of the mesenchymal state[12]. Thus, miR-200 family members promote epithelial differentiation, and their expression is lost in invasive breast cancer cells. The opposing process of MET occurs when the expression of ZEB1 or ZEB2 becomes downregulated because of the loss of EMT-inducing signals; this allows the re-expression of miR-200.

miR-200 can also epigenetically regulate E-cadherin by targeting SUZ12 protein expression[51]. In breast CSCs, the loss of miR-200 increases SUZ12 expression, which results in the polycomb-mediated repression of the *CDH1* gene and upregulation of *ZEB1* and *ZEB2* (ref. 51). The connection between microRNAs and epigenetic regulators is further observed with the histone deacetylase SIRT1 and miR-200. The TGF-β–driven EMT of mammary epithelial cells can upregulate SIRT1 expression, which epigenetically silences the *mir-200* promoter through histone deacetylation[99]. SIRT1 and miR-200 seem to participate in a negative feedback loop, as miR-200 targets the 3¢ untranslated region of SIRT1.

MicroRNAs that contribute to phenotypic plasticity are themselves subjected to epigenetic regulation[97,159–162]. Epigenetic inactivation by CpG island hypermethylation stably silences the *mir-200* promoter, as is observed in highly invasive non–small cell lung cancers that are resistant to chemotherapeutic agents, as well as in invasive bladder and breast cancers[97,160,161]. Interestingly, a stepwise epigenetic repression of the *mir-200* promoter, first through the gain of H3K27me3 and then through DNA methylation, has been observed in bronchial epithelial cells that were dedifferentiating in response to carcinogen exposure[162]. Treatment with a DNA demethylating agent could alleviate *mir-200* promoter hypermethylation and promotes epithelial redifferentiation[98,161]. Likewise, the introduction of a miR-200 analog restores the epithelial phenotype, inhibits tumor growth and metastasis and confers chemosensitivity to otherwise resistant cells[97,159].

One potential strategy for preventing cancer metastasis could involve sensitizing CSCs to traditional therapies by forcing their differentiation into an epithelial state using epithelial-specific microRNAs such as miR-200, miR-34 or let-7. Although microRNA therapeutics are being increasingly explored as options for cancer management[163], the delivery of these molecules to solid tumors still represents a formidable barrier.

Nevertheless, increasingly innovative methods of synthesizing, packaging and delivering these nucleic acids may potentially address such challenges[164].

More recently, long noncoding RNAs (lncRNAs) have been found to affect tumorigenesis and cancer progression largely through epigenetic regulatory mechanisms[165,166]. For example, enforced expression of the lncRNA HOTAIR was sufficient to promote breast cancer metastasis, doing so by associating with PRC2 and modulating PRC2 and H3K27me3 localization to certain sites across the genome[167,168]. Given the increasingly prominent roles of lncRNAs in the epigenetic control of gene expression, it is likely that they will have new functions in regulating cell-state transitions.

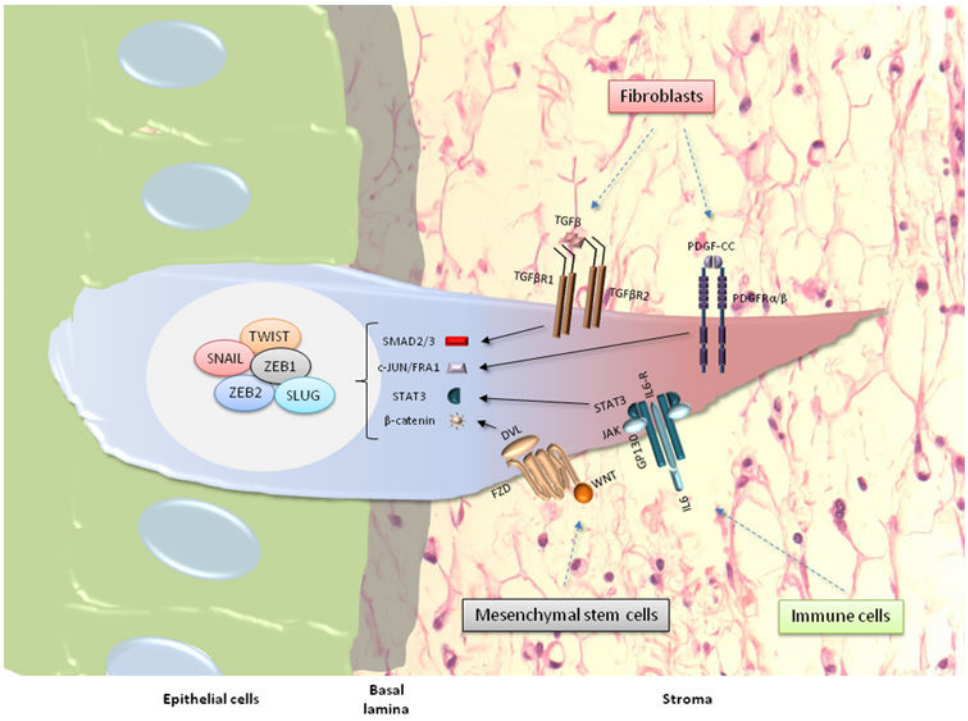

Figure 1.

Connecting extracellular signals to EMT transcription factors. Contextual signals, such as TGF-β, WNT proteins, platelet-derived growth factors (PDGFs) and interleukin-6 (IL-6), arising from autocrine or paracrine signaling networks can activate intracellular signaling factors that influence the activation or maintenance of the EMT transcription factor network during an EMT. TGF-βR1 and TGF-βR2 are two TGF-β receptors; PDGF-CC is a specific member of the PDGF family; PDGFR-α/β indicates two distinct receptor serine/threonine kinases; STAT3 is signal transducer and activator of transcription 3; IL-6R is the IL-6 receptor; gp130 is a membrane glycoprotein; SMAD2/3 indicates SMAD2 and SMAD3; c-JUN/FRA1 are heterodimeric subunits of the AP-1 complex (please note that AP-1 has been defined earlier in Box 1); NK cells are natural killer cells.

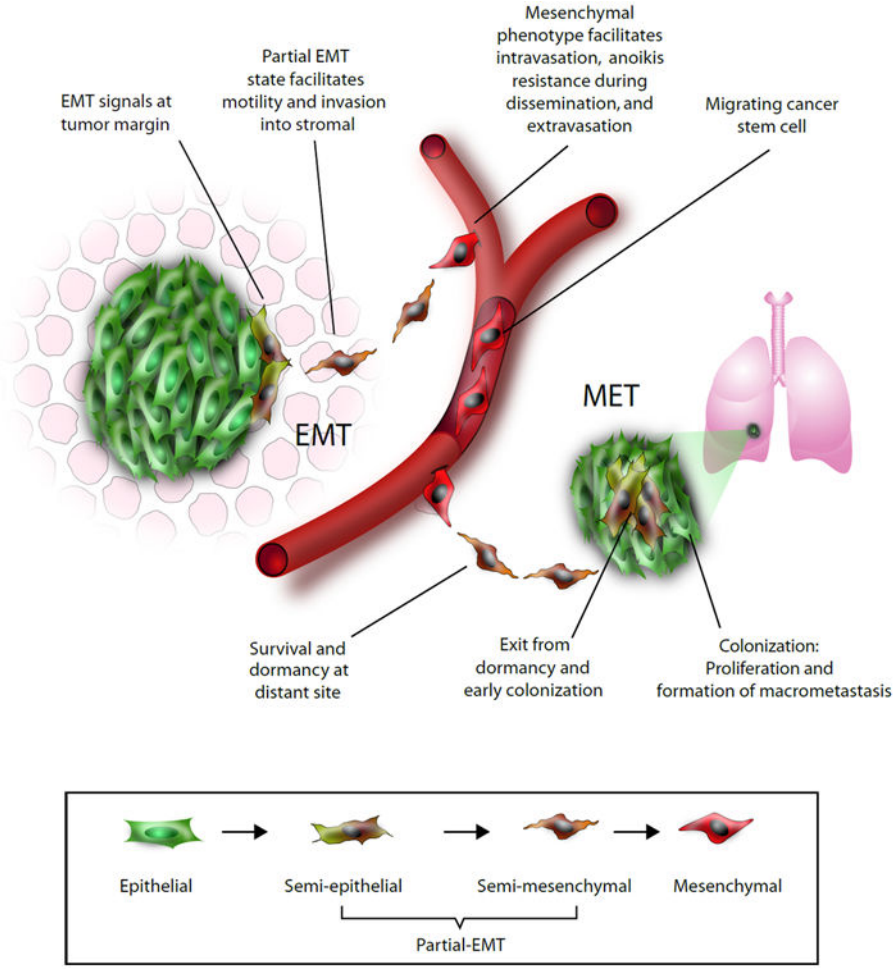

Figure 2.

Epithelial-mesenchymal plasticity allows cancer cells to undergo functional adaptations during the invasion-metastasis cascade. In response to EMT-promoting signals, a subpopulation of epithelial cells at the invasive edge of the tumor may lose epithelial traits. As these cells detach further from the bulk of the tumor, they become less exposed to epithelial signals and acquire more mesenchymal properties in the presence of EMT signals supplied by stromal cells[121–124]. The metastable mesenchymal cells are suited for invasion into surrounding tissues. A fully mesenchymal phenotype facilitates intravasation into blood capillaries or draining lymphatic vessels. In some instances, this process may be aided by macrophages[125]. The disseminating cancer cell is also more resistant to environmental and genotoxic stresses, a characteristic that is crucial for survival in circulation[126]. After arrival at a distant organ, the mesenchymal phenotype facilitates extravasation and invasion into the foreign tissue. Here disseminated cells are exposed to signals different from those of the primary tumor, and the mesenchymal state may confer survival advantages to single cancer cells or alternatively may support long-term dormancy[127]. When the appropriate contextual signals become available, disseminated cells may undergo an MET and gradually reacquire epithelial properties such as rapid proliferative capabilities[16,17]. Epithelial signals are reinforced through autocrine and paracrine signals, resulting in the stabilization of an

epithelial phenotype. This facilitates the outgrowth of macrometastases that are composed predominantly of epithelial cells.

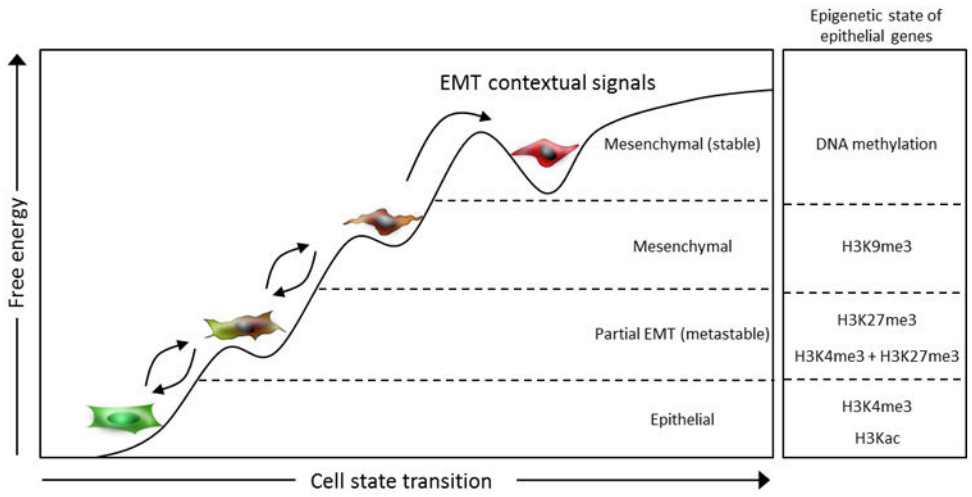

Figure 3.

The epigenetic landscape governs the stability of epithelial-mesenchymal plasticity. The epithelial phenotype is a default state of epithelial cells. Contextual signals promote the epigenetic repression of key epithelial genes (for example, that encoding E-cadherin) by introducing histone modifications, which help define the plasticity of epithelial cells and the residency of cells in a given phenotypic state during the transition. The gain of an increasingly stable mesenchymal phenotype depends on the sustained presence of potent EMT-promoting signals. In their absence, metastable mesenchymal cells may simply revert to a more epithelial phenotype unless they are supported by the appropriate epigenetic modifications. H3Kac, histone H3 lysine acetylation.

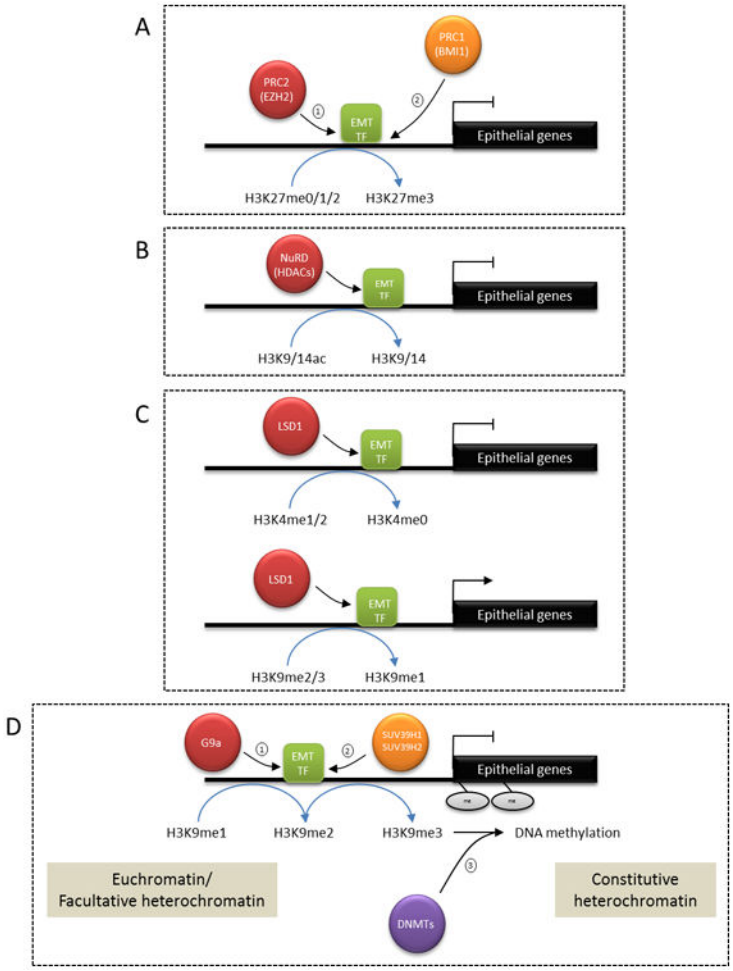

Figure 4.

Interactions between transcription factors and epigenetic regulators. (**a**) PRC1- and PRC2-mediated silencing of epithelial genes such as that encoding E-cadherin involves the initial recruitment of an EMT-TF (for example, SNAIL) to the gene promoter. SNAIL recruits PRC2, which catalyzes conversion to the repressive H3K27me3 mark that is recognized by the PRC1. (**b**) EMT-TFs repress gene activity through the deacetylation of gene promoters. EMT-TFs associate with the NuRD complex, which contains HDACs that catalyze the removal of acetyl groups from lysine residues of histones. H3K9/14, histone H3 lysine 9 and lysine 14. (**c**) SNAIL-mediated recruitment of LSD1 to target genes can result in opposing functional outcomes. LSD1 may catalyze the removal of methyl groups from H3K4, resulting in the loss of transcription activation, or cause the conversion of repressive H3K9me2 or H3K9me3 to H3K9me1, thereby permitting transcriptional activity in conjunction with additional epigenetic modifications. H3K4me1/2, methylation or dimethylation of H3K4. (**d**) SNAIL mediates stable silencing by recruiting G9a and SUV39H1, which cooperatively result in the trimethylation of H3K9. The H3K9me3 mark is a prerequisite for the consequent recruitment of DNMTs, which leads to CpG methylation of gene promoters. The conversion of euchromatin or facultative heterochromatin to

constitutive heterochromatin stably blocks transcription activity. SUV39H1/2, SUV39H1 and SUV39H2.

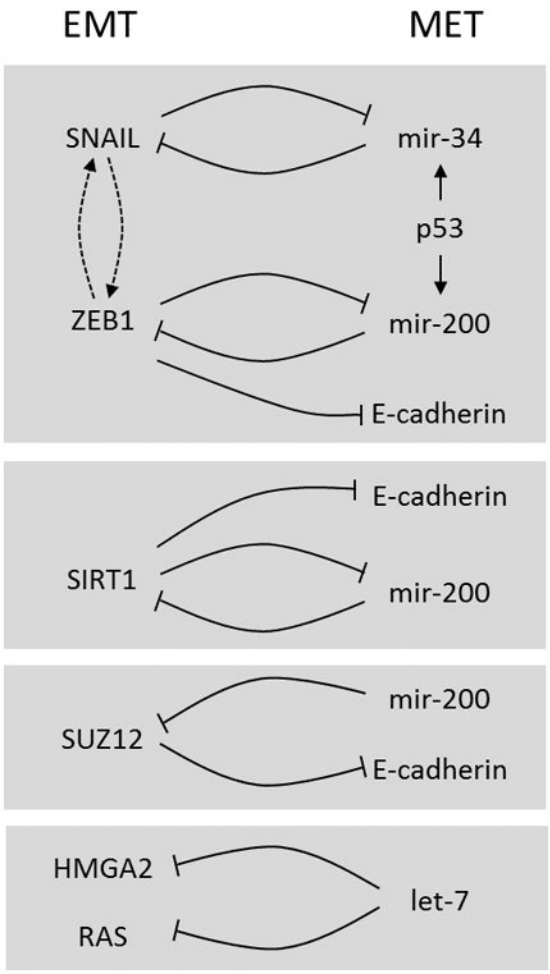

Figure 5.

Integral microRNA transcription regulator networks control epithelial-mesenchymal plasticity. MicroRNAs such as miR-34, miR-200 and let-7 promote the EMT or MET by interacting with certain transcription factors and epigenetic regulators[10,12,51,99,128–130]. Reciprocal negative feedback loops appear to be a common feature that regulates the bi-stable residence of cells in two distinct states.